University of Alberta

Recovery, detection and sanitizer susceptibility of *Listeria* spp. from meat processing environments

by

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©

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta
Fall 2007

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Your file Votre référence
ISBN: 978-0-494-33286-3
Our file Notre référence
ISBN: 978-0-494-33286-3

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DEDICATION

To my parents, Milena and Gojko Kovačević

ABSTRACT

The occurrence of *Listeria* spp. in two meat processing facilities was investigated. Samples were collected in the processing environment of the facilities with different sampling methods (cotton swab-CS, sponge-SS, and composite tissue-CT) to evaluate their ability to recover *Listeria* spp. Four detection assays [culture, environmental *Listeria*-(EL) Petrifilm™, lateral flow immunoprecipitation (LFI), and automated polymerase chain reaction (BAX®)] were evaluated for identification of *Listeria* spp. Persistent and sporadic strains of *L. monocytogenes* were tested for their susceptibility to sanitizers. Occurrence of *Listeria* spp. in the facilities varied from 0.6 to 38%. *Listeria* spp. were isolated from more samples using the SS and the CT ($p<0.01$) than the CS. LFI and BAX® were highly sensitive (95.5% and 99.1%, respectively) and specific (100%) compared to the culture method. Poor performance was observed for the EL-Petrifilm™ with lower sensitivity (50.6%) and specificity (91.5%). No correlation was observed between susceptibility to sanitizers and strain persistence; however, adherence of cells affected sanitizer efficiency.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Dr. Lynn M. McMullen for all her help and guidance throughout my graduate studies.

I thank Dr. Micheal Gänzle, my supervisory committee member and Dr. Monika Keelan, my examining committee member for providing support and assistance for this thesis.

I am grateful to Dr. Valerie Bohaychuk at the Food Safety Division, Alberta Agriculture and Food, for her friendship, invaluable contributions to my research, all the encouragement and the conference adventures. I would also like to thank my colleagues in Food Microbiology for their assistance and patience with my peculiar projects. A sincere thanks to co-workers in Molecular Biology for the use of their laboratory equipment and technical assistance.

I appreciate the friendship of fellow graduate students in the food microbiology lab and all my friends from AFNS for making student life truly enjoyable. I especially thank Kevin Webster for his advice and friendship.

I would also like to acknowledge and thank Dr. Linda Tucker for her help and encouragement. Without her, I would not be where I am today.

Finally, I would like to express my extreme gratitude and love to my parents, my sister and Jared for all their help and support throughout the years. Their influence and encouragement provided great inspiration in all my academic achievements.

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1. INTRODUCTION AND LITERATURE REVIEW

The presence of pathogenic microorganisms in food processing facilities is of great concern to food processors, consumers and health authorities *(59)*. Consumer demand for minimally processed foods with assurances of a safe product has resulted in an increased demand for adequate testing, cleaning and sanitation of food processing, distributing, and retail facilities. Of special concern are those bacteria that can adhere to food contact surfaces and form biofilms *(156)*. Once the bacteria adhere to surfaces and form biofilms, they become more difficult to remove *(96)*. In addition, it has been observed that susceptibility to sanitizers decreases when bacteria are a part of a biofilm consortium *(98, 107)*.

One of the common biofilm formers that inhabit food processing environments are *Listeria* species. *Listeria* can persist in processing environments for extended periods of time and potentially result in continuous contamination of food products *(73, 74, 96)*. Foods such as processed meats have been implicated in numerous listeriosis outbreaks *(14, 30, 98)* which resulted in increased regulations and closer monitoring of meat processing facilities *(68, 145)*. Due to the high risk associated with contamination of processed meats, it is imperative that food processors understand the potential sources of contamination, distribution and characterization of persistent bacterial strains, as well as which measures are effective in control of *Listeria* spp. in the food processing sector.

To achieve successful monitoring and control of a pathogenic bacterium such as *Listeria monocytogenes,* it is important that all steps involved in the evaluation of food safety are addressed. Regular sampling of foods and the processing environment is imperative. The food industry has been provided with numerous sampling methods and a variety of analytical products; however, some methods may be more efficient in recovery of specific pathogens than others. Currently, there is no standardized protocol for environmental swabbing and a wide range of materials such as cellulose sponges, composite ply-tissues and cotton tipped swabs are commercially available. However, the lack of comparable data for the swabbing protocols makes it difficult to evaluate the efficacy of each sampling method.

Once environmental sampling is conducted, it is extremely important that appropriate detection methods be used to achieve reliable and rapid results. Prompt results are of paramount importance to the food industry where a timely response to potential food safety issues is critical. With the understanding of the source and distribution of *Listeria* spp. in meat processing facilities, more appropriate monitoring and control measures can be achieved.

1.1 Characteristics of the genus *Listeria*

Listeria are Gram-positive, non-sporeforming short rods. They are motile, catalase-positive, oxidase-negative, and utilize glucose with acid being the end product of glycolysis *(123).* Genus *Listeria* is comprised of six different species: *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi (36, 71). Listeria murray* was originally regarded as a separate species of

Listeria; however, in 1992 the description of *L. grayi* was revised and *L. murray* was reassigned to *L. grayi (130)*. Among the six species, *L. monocytogenes* has been recognized as both a human and animal pathogen, capable of causing a variety of infections in humans, domesticated and wild birds and mammals *(149)*. *L. ivanovii* has been known to cause disease in animals and very rarely in humans *(36, 71)*. *L. seeligeri* is considered one of the avirulent strains; however, due to detection of many pathogenic traits carried by *L. seeligeri* it has been reported that this particular strain is capable of causing disease in humans *(61, 84, 149)*.

L. monocytogenes is both a facultative anaerobe and a facultative intracellular pathogen as well as a known causative agent of an uncommon but potentially dangerous illness called listeriosis *(123, 149)*. Due to its ability to survive adverse environmental conditions, *L. monocytogenes* has become a notable villain in the food industry *(84)*. A variety of foods such as dairy, meat, egg, seafood and vegetable products *(51)* have been reported as vehicles of transmission in cases of listeriosis. One of the characteristics of this microorganism that plays a crucial function in its role as a foodborne pathogen is its ability to survive and grow at wide range of temperatures (i.e. between -0.4 °C and 50 °C) including the refrigeration temperatures *(149)*. In addition, *L. monocytogenes* can survive a broad range of pH conditions and osmotic pressures *(149)*. These traits have added greatly to the difficulty of keeping *L. monocytogenes* outside of the food chain.

Although descriptions of the microorganism, which is now known as *L. monocytogenes*, date back to 1911 when the bacterium was isolated from necrotic foci of a rabbit's liver *(64)*, it was not until 1924 that Murray et al. *(114)* isolated and described the bacterium as *Bacterium monocytogenes*. In the following years a number of different names were assigned to the bacterium until 1940, when Pirie *(125)* named it *Listeria monocytogenes*. Historically, as an animal pathogen, *L. monocytogenes* was commonly found in rabbits, guinea pigs, sheep and cattle *(64)*. However, in the last three decades *L. monocytogenes* has emerged as an important foodborne pathogen *(132)* that has caused outbreaks with high morbidity and mortality rates *(132)*.

As a result of the many unique features of *L. monocytogenes*, extensive research has been done regarding its virulence traits and mechanisms of invasion. With the use of novel genetic techniques and tools, as well as variety of *in vitro* models used for infection, *L. monocytogenes* has become one of the model pathogens for studying the basis of intracellular parasitism *(61)*. The identification of genes that play an important role in the pathogenesis of *L. monocytogenes* has led to detailed findings about the cell infectious process *(61)*.

1.2 *Listeria monocytogenes* pathogenesis and listeriosis

As an intracellular pathogen, *L. monocytogenes* has evolved a number of mechanisms to take advantage of host cell processes, enabling it to grow and spread in the human body without causing toxicity to the host cell *(126)*. These bacteria have the ability to invade and proliferate within macrophages and a

range of non-phagocytic cells, including epithelial, endothelial, and hepatocytes *(148)*. The most common point of entry into a human body is the gastrointestinal route, through consumption of contaminated foods *(149)*. However, there has been some controversy pertaining to the entry point of the bacterium into the host cells, depending on the infection models used. Studies with guinea pigs support the theory that *L. monocytogenes* penetrates the host cell through the intestinal villous epithelium *(127)*. If mice are used as models, bacteria preferentially colonize Peyer's patches through the use of the M-cell epithelium rather than invading the intestinal villous epithelium *(137)*.

Once *L. monocytogenes* adheres to and successfully enters the host cell, the intracellular endurance is dictated by its ability to avoid the immune system response through the use of highly sophisticated survival and virulence mechanisms *(51)*. The expression of the specific virulence genes is highly dependent on the stage of bacterial life cycle, as well as the location of the bacterium inside a host *(28, 46, 113, 148)*.

A number of different opsonin- dependant and opsonin- independent mechanisms play a role in internalization of *L. monocytogenes* into phagocytes *(148)*. The bacterium induces its own uptake by nonprofessional phagocytes through a variety of mechanisms involving invasion proteins: InlA, InlB, and P60 *(148)*. After internalization of the bacterium, *L. monocytogenes* goes through a cycle of events including "escape from the phagocytic vacuole, multiplication in the host cell cytoplasm, directional intracytosolic motility by induction of actin polymerization at one pole of the bacterial cell, protrusion of centrifugally moving

bacteria within cytoplasmic evaginations, and phagocytosis of the pseudopod-like structures by neighboring cells" *(148)* which can be repeated a number of times.

Regardless of the cell type in which *L. monocytogenes* finds itself, internalization is rather quickly followed by lysis of the phagocytic vacuole, and release of the bacterium into the cytosol where it can undergo cell replication and spread into adjacent cells *(37)*. *L. monocytogenes* can easily be set free into the cytosol and by means of actin-polymerization it can be propelled from one cell to the other, resulting in easy dissemination from the small intestine to the liver, spleen, the brain stem, and the placenta *(37, 45, 149)*. Depending on the point of entry of the bacterium and the type of a host cell, different bacterial surface proteins are employed. For instance, Internalin A (InlA) protein is known to play a role in the invasion of the human enterocyte-like Caco-2 cells, while Internalin B (InlB) is involved in invasion of Vero, HeLa, Hep-2 and CHO cells *(21, 75, 93, 158)*. A variety of factors are important in this initial stage of internalization; however, motility and adherence are imperative for the bacterial survival of adverse conditions of the stomach and efficient uptake into the cell, respectively *(149)*.

A wide variety of infections associated with listeriosis have been attributed to the ability of *L. monocytogenes* to easily adapt to the environment in which it finds itself. The ability of different *L. monocytogenes* serotypes to cause more or less serious infections also depends on the bacterial adaptation as well as the state of the immune system of a host *(149)*. The specificity of the serotypes to the distinct disease symptoms, and particular sources (i.e. human, food, and

environmental sources), further proves that complex relationships and interactions are responsible for differences in virulence and survival of *L. monocytogenes* in different milieus *(95)*.

As a causative agent of an uncommon but potentially severe illness, listeriosis, *L. monocytogenes* has been the cause of many costly outbreaks throughout the world *(5, 135, 139)*. However, sporadic cases of *L. monocytogenes* infections tend to be the more common occurrence *(147, 149)*. Listeriosis can manifest itself in different forms, some of which include diarrhea, meningitis, meningoencephalitis, spontaneous abortions, still births, septicemia, as well as systemic infections in neonates, pregnant and immunocompromised individuals *(51, 149)*. Unlike most other foodborne infections, listeriosis is known for its severity, and high hospitalization and case fatality rates *(58)*.

Risk assessments of *L. monocytogenes* indicate that humans are frequently exposed to the bacteria *(51, 128)*. In fact, it is believed that 5 to 10% of human population are carriers for *L. monocytogenes* without exhibiting the illness *(51, 149)*. Typically, to contract listeriosis several simultaneous events need to occur, such as exposure to large numbers of microorganisms with concurrent infringement of the intestinal barrier and decreased nonspecific defenses and immune system response *(51)*. Based on the documented outbreaks throughout the world mortality rates associated with listeriosis have been reported anywhere from 13 to 45% *(51, 128)*. Neonates, immunocompromised individuals, elderly and people with central nervous system infections are known to exhibit the most severe consequences of the disease *(80, 128)*.

Initially *L. monocytogenes* was regarded as an animal pathogen without much connection to the foods. However, in 1981 an outbreak involving coleslaw in Canada, provided scientific evidence for the first time that *L. monocytogenes* contaminated the food and that food was the route of transmission for the organism *(128)*. Detailed investigations of outbreaks further confirmed the idea, and in 1988 the World Health Organization acknowledged that foods are a major vehicle of transmission for the spread of *L. monocytogenes (128)*. Furthermore, it is believed that food contamination in food processing facilities can occur as a result of persistence of *L. monocytogenes* in food handling areas *(14, 30)*.

1.3 Foods as vehicles for *L. monocytogenes*

The ease of transmission of *Listeria* from the environment, animals and food contact surfaces has resulted in numerous food outbreaks throughout the world *(35, 50, 87, 139, 141, 146)*. A variety of vegetables *(15)*, dairy foods *(83)*, seafood *(110)*, meat and poultry products *(76)* have been implicated in outbreaks of listeriosis that caused costly recalls and had devastating health concerns.

The first documented food outbreak involving *L. monocytogenes* occurred in the province of Nova Scotia in 1981 *(133)*. One of the first foodborne outbreaks described, involved consumption of coleslaw contaminated with *L. monocytogenes (59)*. The source of contamination was attributed to fertilization of cabbage with raw manure from mastitic sheep infected with *L. monocytogenes (133)*. Overall, 41 people were infected, resulting in 17 deaths *(133)*. Following the outbreak, numerous surveys and testing of produce worldwide revealed that vegetables such as bean sprouts, cabbage, cucumber, potatoes, radish, tomato,

and leafy vegetables can harbor *L. monocytogenes (15)*. In 1988, Sizmur and Walker *(138)* tested 60 samples of various salads from local stores, containing bean sprouts, cabbage, celery, sultanas, onion, carrots, lettuce, cucumber, radish, fennel, leeks, and watercress. *Listeria monocytogenes* was isolated from four salads, while *L. innocua* was found in 13 samples *(138)*. However, in the late 1980's, surveys performed in the United States *(124)* and Canada *(52)* which involved testing of lettuce, celery, tomatoes and radishes, found no detectable *L. monocytogenes* in any of the vegetable samples. Outbreaks involving *L. monocytogenes* with produce have not been common; however, products such as potato salad have been frequently recalled in various American states due to suspected *Listeria* contamination *(2, 7)*. Even though *Listeria* outbreaks from consumption of vegetables are not commonly observed, the potential for contamination and illness still exists. In 2000, multiple cases of febrile gastroenteritis were observed in 72% of individuals exposed to a contaminated cold salad of corn and tuna in two Italian towns *(10)*. Experimental analysis revealed that when stored at 25°C for at least 10 hours, corn supported the growth of *L. monocytogenes (10)*.

In addition to produce, contaminated dairy, seafood, and meat products are important vehicles of transmission for *L. monocytogenes*. Outbreaks involving pasteurized whole or 2% milk *(54)*, Mexican-style fresh cheese *(8, 94)*, soft cheese *(22, 24, 62)*, butter *(91, 100)*, and whipping cream *(121)* have been reported in European as well as North American countries. In an outbreak

involving soft cheese consumption in western Switzerland, 57 cases of listeriosis were observed, with 32% overall mortality *(22)*.

Contrary to produce and dairy products, where contamination is mostly from animals and farm environment *(15, 16, 81)*, in the case of meat products, contamination is most frequently associated with production facilities and post-processing handling *(30, 48, 115)*.

Based on the data collected from 1995 until 2000, The Canadian Listeriosis Reference Service (LRS) reported approximately 47 to 79 cases of human listeriosis per year *(121)*. Lee and Middleton *(90)* conducted a study from 1997 to 2001, investigating sporadic cases of illness in Ontario due to eight enteric pathogens. They found that 74.0% of the outbreaks were foodborne. Furthermore, poultry and other meat products accounted for 68.4% of foods responsible for illness *(90)*. Although the incidence of listeriosis was less than 5 cases per million people, the hospitalization (47.1%) and case-fatality (23.8%) rates were the highest for *Listeria (90)*.

Eleven states in the United States reported at least 50 listeriosis related illnesses from August 1998 through January 1999 *(27)*. Hot dogs and deli meats from one manufacturer were identified as the contaminated products in all the cases. In this multistate outbreak six adults died and two pregnant women had spontaneous abortions *(27)*. Similarly, in 2002, an outbreak involving consumption of contaminated turkey deli meat resulted in 46 confirmed cases of listeriosis, of which seven people died and three stillbirths or miscarriages occurred *(25)*. Eight states in the Northeastern part of the United States were

affected by this outbreak. Testing of the intact food product and the processing environment discovered the presence of *L. monocytogenes (25)*. Furthermore, genetic fingerprinting analysis showed different patterns for the *L. monocytogenes* found in the food sample and for the outbreak strain; however, two environmental isolates obtained from floor drains were genetically indistinguishable from the outbreak strain, indicating that the contamination of the food most likely came from the processing environment *(25)*.

Although data reported by the Foodborne Diseases Active Surveillance Network (FoodNet), and Centers for Disease Control and Prevention indicate a decreasing trend for overall incidence of listeriosis in the United States in comparison to the rates observed from 1996 through1998, the incidence observed from 2003 through 2006 remained higher than at its lowest point in 2002 *(26)*. In Canada, listeriosis has been a reportable disease since 1990 and generally low infection rates are reported. In 1998 and 1999, rates of 3.4 and 2.5 cases per million people, respectively, were reported *(1)*. Specifically in Alberta, the rate of 4.1 cases per million people was observed in 1998 and 1999 *(1)*.

Through education, strict regulations and effective monitoring, the food industry and government authorities throughout the world have resolved to reduce *Listeria* contamination of foods, as well as to relieve the economic burden that *Listeria* has imposed on society. Although the occurrence of listeriosis can still be observed throughout the world, it has been noted that wherever active surveillance is in effect, decreasing rates of listeriosis are recorded *(134)*.

1.3.1 Incidence of *L. monocytogenes* in food processing facilities

Food production areas are favorable for the growth of *L. monocytogenes* due to the constant flow of water and food ingredients. Contamination of ready-to-eat products with *L. monocytogenes* has been linked to contamination of food processing equipment and food production environments *(30, 97)*. Due to the high health risk associated with the presence of *L. monocytogenes* in cooked foods, numerous studies have focused on the assessment of overall prevalence and contamination patterns in food processing facilities. Furthermore, investigations of the possible transfer of *L. monocytogenes* from the food processing environment and equipment to raw, as well as finished food products, have been the focus of many scientists.

In a study conducted by Lawrence and Gilmour *(88)*, the incidence of *Listeria* spp. and *L. monocytogenes* in a poultry processing environment and products was investigated over a six-month period. *L. monocytogenes* was found in 26% of the samples obtained in the area processing raw poultry and 15% of the samples were recovered in the area where cooked poultry products were handled *(88)*. Overall, 59% of the raw poultry products were contaminated and while no *L. monocytogenes* were detected in cooked products, 8% were positive for other *Listeria* spp. *(88)*. In addition, some locations in the processing environment were consistently positive for the presence of *L. monocytogenes*, indicating that although none of the tested cooked products were positive for the bacterium, the potential for contamination existed. Similar trends have been

observed in other studies that linked outbreaks with the heavy contamination of a food processing environment.

In an investigation of a nationwide outbreak in the United States that occurred from July 1998 until April 1999, meat frankfurters and deli meats were identified as the likely source of *L. monocytogenes* infection *(105)*. The total number of patients with listeriosis was 108, which led to 14 deaths and four miscarriages or stillbirths *(105)*. One food processing establishment was identified as the source of *L. monocytogenes* outbreak and further testing of the products collected from the facility confirmed the presence of two serotypes, 4b, which was associated with the outbreak, and 1/2a, which was present in high numbers in the product but was not associated with the infections *(105)*. Furthermore, it is believed that the outbreak strain had colonized the ceiling refrigeration unit in one of the rooms where meat was exposed and that removal of the ceiling refrigeration unit led to increased spread of the bacterium onto production equipment, environmental surfaces, as well as meat products *(105)*. Increases in the number of swabs that tested positive for psychrophilic organisms following the removal of the refrigeration unit further proves this theory *(105)*.

Similarly, in 2002 consumption of turkey deli meat contaminated with *L. monocytogenes* was the cause of an outbreak involving 46 confirmed cases; three stillbirths or miscarriages and seven deaths *(25)*. It was speculated that the food processing environment was the source of *Listeria* contamination since the outbreak strain and isolates collected from the floor drain shared an indistinguishable Pulsed-Field Gel Electrophoresis (PFGE) pattern *(25)*.

Jemmi et al. *(77)* evaluated the prevalence of *L. monocytogenes* in 425 meat and fish production facilities over a nine-year period. The pathogen was found in 20.5% of the facilities, of which most had problems with adapted in-house strains that were difficult to eliminate *(77)*. Adapted strains have been observed by Chasseignaux et al. *(30)* for one year in a poultry processing plant and for four months in a pork processing facility. A year and a half survey of a smokehouse in Denmark indicated that one dominant strain persisted in the environment and accounted for 80% of the isolates *(157)*. Furthermore, in a three-year study of the microbial ecology of high-risk, chilled food factories producing a large range of ready-to-eat meats, Holah et al. *(73)* observed 14 *Listeria* spp. ribogroups repeatedly recovered from the same locations in the processing environment over a prolonged time period.

Not only has persistence of certain *Listeria* spp. been observed in numerous food processing plants, but transfer of these strains from the food processing environment and equipment to food products has also been demonstrated *(92, 97)*. Lin et al. *(92)* conducted a study to investigate cross-contamination between processing equipment and deli meats by *L. monocytogenes* and showed that the bacterium can be transferred from a contaminated deli slicer onto meats. Furthermore, contamination of the meat was correlated with the initial numbers of *L. monocytogenes* on the slicer, with oven-roasted turkey products harboring the highest numbers of bacteria due to rapid growth during refrigeration *(92)*. Their findings also suggest that the degree of transfer of *L. monocytogenes* is product dependent, while the detection of the

organism is dependant on the sample size *(92)*. Therefore, due to the generally low numbers of *Listeria* that are present in food *(76)* and a possible localization in a sample *(92)*, it is likely that the number of food samples that test positive for *L. monocytogenes* is often underreported.

Similarly, Lundén et al. *(97)* demonstrated that a dicing machine was a continuous source of *L. monocytogenes* contamination in the food processing area of three different food establishments. Specifically, facility A had problems with one specific PFGE type of *L. monocytogenes* in 1997, after which the dicing machine was transferred to facility B, and then to facility C. Following the removal of the dicing machine from facility A, contamination with *L. monocytogenes* ceased; however, with the move of dicing machine to facility B, and subsequently to facility C, contamination with the same *L. monocytogenes* PFGE type started to emerge in the facilities *(97)*. The dicing machine used in these facilities is considered to be a complex type of the equipment with certain areas that are difficult to reach, clean and properly sanitize. It is believed that these particular areas are ideal for harboring *L. monocytogenes (97)*.

Reasons for prolonged survival of specific *L. monocytogenes* strains on food processing equipment and in the environment are still unclear. Different hypotheses for persistence have been proposed; however, the lack of evidence for any particular theory validates the complexity of the issue. It is believed that a number of prevailing conditions in food processing facilities play a role in plant contamination and persistence of *L. monocytogenes (97)*.

1.3.2 Survival of *Listeria* spp. in food processing environments

Unlike the majority of strains of *Escherichia coli* that are generally product specific *(73)*, it has been observed that persistent *Listeria* spp. are mostly from environmental sources *(73)*. Lundén et al. *(99)* investigated contamination of three meat and one poultry processing facility focusing on the differences in persistent and nonpersistent strains of *L. monocytogenes*. Both types of strains were found in all of the facilities sampled, with nonpersistent strains recovered mostly at one sampling site and persistent strains obtained from a number of different sampling locations *(99)*. Furthermore, persistent strains were typically recovered from the processing machines, with some instances of a final product being contaminated with the same PFGE type of *L. monocytogenes* that was found in the processing equipment *(99)*.

Presence of nonpersistent strains in a food processing facility is believed to be due to entrance via raw materials, personnel, water or air supply *(73)* and these strains are generally eradicated with adequate routine cleaning and sanitation *(143)*. Persistent strains tend to be established in particular environmental niches and are very difficult to eliminate *(73)*. Specific characteristics contributing to persistence of a strain are unknown; however, a few hypotheses have been suggested. Presence of persistent strains in food processing environments, such as floors, drains, carts, racks, and processing equipment, has been attributed but not limited to inadequate personnel education regarding the importance of good manufacturing practices, poor cleaning, improper sanitation, as well as inferior equipment design *(48)*.

Complex processing machines seem to be the main locations harboring *L. monocytogenes*, as certain areas contain micro-spaces in which bacteria can become imbedded and protected from sanitizers *(97)*. Furthermore, the key elements in bacterial survival have been attributed to the formation of biofilms, increased initial attachment, as well as enhanced attachment strength *(73)*. Some studies have also proposed increased resistance to sanitizers as the reason for challenges in eradication of persistent strains of *L. monocytogenes (98)*. Nonetheless, whether it is a physical or genetic adaptation to specific environmental conditions, poor cleaning and sanitation practices, or decreased susceptibility to specific sanitizers, persistent strains seem to be a common occurrence in food processing facilities, and therefore require particular attention.

1.4 Sampling and detection of *Listeria* spp. in the food processing environment

Although the risk of contamination by *L. monocytogenes* can be reduced, the absolute elimination of this microorganism from the food processing environment is hard to achieve. In addition to preventative measures, efficient sampling methods and reliable and rapid isolation and detection assays are extremely important. Sampling of the final food product is generally a priority. However, for pathogens such as *L. monocytogenes*, routine sampling of the processing environment is of equal importance.

1.4.1 Sampling methods

Environmental sampling in the food processing sector tends to vary between different facilities when it comes to materials and procedures. Some of the important factors affecting the choice of a sampling device include the efficiency of a sampling method in its ability to recover specific microorganisms, cost, the time required for assembly or preparation of a sampling kit, and the ease of handling. It has been established that certain conditions, such as wetness or dryness of a surface can impact the ability of a swab to remove bacteria from a surface *(23, 111)*. The material of a swabbing device may have inhibitory substances or undesirable properties that can result in the decreased recovery of specific microorganisms *(39)*. .Some sampling kits, such as sterile ready-to-use sponges, are generally convenient and easy to use; however, certain properties of the sponge material, such as porosity, greatly reduce the recovery of bacteria *(39, 111)*.

Materials such as cotton tipped swabs may be used in a wide variety of sampling applications, including swabbing of beef carcass *(154)*, sampling of the outdoor environment *(23)*, and sampling of food processing establishments *(112)*; however, due to the small surface area of the swab, decreased initial microbial recovery and impracticality for quantitative sampling have been demonstrated *(152)*. Nonetheless, in sampling of difficult-to-reach areas and small surfaces of complex processing machines or surfaces with small openings and crevices, cotton tipped applicators may be more desirable for swabbing than larger sampling devices.

In environments where quantitative microbial sampling is required, there is a need for a larger surface area and a less porous material for the sampling device in order to maximize the initial recovery of the bacteria from the surface of interest and increase the chance of the release of bacteria from the sampling device once the sample is processed. Materials such as one-ply composite tissues have been evaluated by Vorst et al. *(152)* in their ability to recover *L. monocytogenes* from stainless steel surfaces, even when bacteria are present in small numbers. In comparison to a sterile environmental sponge, a sterile cotton-tipped swab, and a sterile calcium alginate fiber-tipped swab, the one-ply composite tissue was superior in recovery of *L. monocytogenes* organisms *(152)*.

The lack of standard for protocols swabbing for environmental sampling of food processing facilities limits the extent of comparison of the prevalence of specific microorganisms in different food processing establishments. Quantitative sampling tends to be complicated due to difficulties in controlling the reproducibility and repeatability of the swabbing techniques *(112)*. Moore and Griffith *(112)* demonstrated that both swabs and wetting solutions can affect microbial recovery. Therefore, when choosing a sampling method, properties of both the sampling device and the wetting solution need to be appropriate for the recovery of the microorganisms of interest, or microorganisms expected to be present *(112)*.

1.4.2 Detection methods

The food industry is striving for reduction and possible elimination of undesirable microorganisms in food; however, when safe manufacturing

practices fail and products become contaminated, there is reliance on methods for the detection of harmful pathogens. Many foodborne outbreaks are underreported *(118, 128, 129, 151)*, nevertheless, in those that are reported and food, fecal or environmental samples are analyzed, rapid and sensitive pathogen detection methods play a very important role.

Generally, in food processing facilities *Listeria* spp. are present in low numbers *(76)*. Furthermore, overgrowth by other *Listeria* spp. is believed to often mask the presence of *L. monocytogenes (109, 144)*. In some instances, *L. innocua* were found to produce bacteriocins capable of inhibiting the growth of *L. monocytogenes* in enrichment cultures *(159)*. For this reason, highly sensitive and specific detection methods are required for testing of food products and environmental samples for the presence of bacteria.

Principles for the conventional culture method date back to the 19th century and are still relied on today *(12)*. Culture methods were the first assays available for detection of pathogenic microorganisms and historically they were regarded as the most reliable methods *(20, 42)*. Culture methods have been considered the "gold standard" for the isolation and identification of *Listeria*, and even now, new technologies are evaluated by comparison with the culture methods *(17, 131)*. A variety of different broths and agar media have been developed for enrichment and selective growth purposes. In the case of *Listeria* spp., *Listeria* enrichment broth (LEB) *(120)*, Half-Fraser broth (HFB), and University of Vermont (UVM) *(4)* broths are commonly used for primary enrichment. Fraser broth (FB) is generally used for selective enrichment following the primary

enrichment *(4, 120)*. Further isolation is typically performed by plating the selective enrichment broth samples onto different solid media. Some of the agars used for identification of *Listeria* spp. are Oxford agar with or without different modifications, PALCAM agar, lithium chloride-phenylethanol-moxalactam medium (LPM), as well as some of the novel chromogenic agars *(120)*, such as Harlequin™ Listeria chromogenic agar, Oxoid (OCLA) *(155)* and ALOA chromogenic agars *(150)*. Presumptive *Listeria* colonies are further purified on different blood media, such as sheep or horse agars, or non-selective media such as tryptose and trypticase soy agars, as well as in some broths (e.g. brain heart infusion). Confirmation of presumptive colonies of *Listeria* can be done by tests for motility, carbohydrate utilization, Gram stain, catalase, CAMP test, rapid identification kits that differentiate *Listeria* species, as well as different serology tests *(4, 120)*.

Numerous improvements of culture methods have been observed; however, the process is still laborious and time consuming, since it relies on the growth rate of microorganisms. Movement towards automation has been seen in systems such as ISO-GRID, Redigel® and Easygel®, SimPlate®, and Petrifilm™, to reduce labor inputs. The ISO-GRID method with LM-137 agar resulted in significantly higher recovery of *L. monocytogenes* from a variety of meat products, pasteurized and raw milk, shrimp, and environmental swab samples *(49)* compared to most probable number procedures with enrichment methods published by the U.S. Department of Agriculture, the Association of Official Analytical Chemists International and the U.S. Food and Drug Administration.

The Petrifilm™ Environmental Listeria plate method has been reported as rapid, accurate, highly specific and sensitive method in a few studies *(65, 160)*, with no significant difference in detection of *L. monocytogenes* recovered from inoculated environmental surfaces when compared to the U.S. Department of Agriculture Food Safety and Inspection Service method *(66)*. Regardless of time and labor drawbacks of culture methods, they are still relatively cost-effective and easy to perform, with the latter characteristics being the key factors for some food industries *(63)*.

Nonetheless, for products with a short storage life there is a need for rapid pathogen detection methods so that prompt results and a timely response to potential food safety issues can be achieved. As a result, a great deal of research has focused on the development of rapid and reliable technologies for detection of pathogens in both food and environmental samples. Presently, pathogenic microbes can be detected using biochemical, physicochemical, as well as nucleic acid based technologies *(142)*.

The ability to amplify very small amounts of specific DNA sequences, using various polymerase chain reaction (PCR) methods has revolutionized the way pathogens are detected and analyzed. With this enormous leap into the next technological phase there is also a challenge of how to integrate the innovative methods into everyday use in food processing facilities. Foods are known to be complex and vary in their composition, which can subsequently have an effect on these novel technologies. These challenges need to be carefully considered when choosing the appropriate pathogen detection method. In fact, the methods

need to be able to detect the pathogen of interest in the presence of other microorganisms and food components. Hindrances, such as a competitive microflora and food particles, are especially prominent in environmental samples obtained from food processing facilities. Detection methods often need to be able to detect very small numbers of target pathogens in a short amount of time *(34)*.

Nucleic acids, such as DNA and RNA, are used in genetic techniques for detection of pathogenic microorganisms due to the fact that the specific sequence of the target gene can give the information required to identify the type of microorganism it came from *(70)*. In contrast to most conventional detection methods, PCR does not always require the viability of microorganisms, therefore, culturing of the bacteria is frequently omitted. As a result, the time factor is significantly decreased *(142)*. In addition, a minimal amount of material is required for identification, with detection capabilities of as low as one molecule of target DNA *(142)*. The BAX® system by DuPont Qualicon, is an example of an automated PCR system, that can be used for the detection of various microorganisms. Studies have reported sensitivity rates ranging from 84.8% *(72)* to 100% *(13)* for testing *L. monocytogenes* in raw fish materials and cold smoked vacuum packed salmon, respectively. Similarly, specificity rates have been observed from 84.9% *(131)* for detection of *L. monocytogenes* in equipment swabs from a chicken nugget processing facility to 100% *(72)* when raw fish samples are tested.

Use of other types of PCR-based methods for detection of *Listeria* spp. have also been reported in the literature as excellent alternatives to culture methods. Lawrence and Gilmour *(88)* evaluated a multiplex PCR technique for confirmation of *Listeria* spp. and *L. monocytogenes* in poultry and environmental samples obtained from food processing areas and concluded that this method is highly convenient, specific, and rapid for detection of Listeriae. For naturally contaminated samples obtained by swabbing environmental surfaces in food processing environments, due to generally low numbers of *L. monocytogenes* present, an enrichment step may be necessary *(11)*. In a study performed by Aznar and Alarcón *(11)*, *L. monocytogenes* had to be present in a sample in at least 10^3 CFU g^{-1} to be detected by PCR following a DNA extraction method. Although PCR-based methods are generally sensitive and rapid, at least one type of enrichment prior to the analysis is desirable followed by confirmation protocols *(53)*. Additionally, highly skilled personnel are typically required to perform the tests, which may be a drawback for the routine use in the food industry setting.

In addition to PCR assays, specific antibodies that target surface antigens can be used as means of detection of different bacterial pathogens *(34, 153)*. By labeling antibodies with fluorescent molecules or radioisotopes, bacteria can be successfully detected in food and environmental samples *(34)*. Some of the common methods based on the antibody techniques are immunoprecipitation and immunofluorescent methods, enzyme linked immunosorbent assay (ELISA), as well as different variants of ELISA methods *(34)*.

The Reveal® test is an example of a combination of technologies for the detection of bacteria in different samples. In addition to immunoassay, chromatography is incorporated into lateral flow devices to enable rapid and accurate results in one step *(6)*. Similarly, the RapidChek™ *Listeria* lateral flow test uses a double antibody sandwich format based on antibodies specific to *Listeria (9)*. If *Listeria* spp. are present the antibody complexes form resulting in a colored line in the test window of the device *(9)*. In Oxoid's *Listeria* Rapid Test kit- Clearview™, flagellar antigen B common to *Listeria* species, with the exception of *L. grayi*, is used to identify the presence of *Listeria* in a sample *(153)*. The test device of the Clearview™ system is similar to Reveal® and RapidChek™ where a colored line in the test device window indicates a positive result. Rodrigues et al. *(131)* reported sensitivity and specificity values of 98.5% and 100%, respectively, for the *Listeria* Rapid Test Clearview™ for detection of the bacteria in product and environmental samples obtained from a chicken nugget processing plant.

Testing of the processing environment for the presence of *Listeria* spp. is a relatively new development and although a variety of rapid detection methods exists, the lack of direct comparison of the technologies makes it difficult for food producers to decide on the most appropriate and practical method to meet the needs of their facility.

1.5 *Listeria* biofilms

Biofilms are communities of microorganisms attached to a surface and embedded in a protective and adhesive matrix of extracellular polysaccharides of

their own making *(119)*. These complex aggregations of microorganisms have been observed on numerous surfaces such as living tissues, medical devices, industrial and potable water pipes, as part of natural aquatic systems, as well as on floors, drains, pipes, seals, conveyor belts, and almost all surfaces found in food processing facilities *(86, 119)*. Similar to human communities, bacterial biofilms are very complex in nature. Biofilms can be comprised of a single microbial species, which is often the case with medical devices biofilms, or they can involve multiple microbial species living with each other (i.e. biofilms found in environmental systems). However, regardless of the number of species involved, all biofilms have been observed to exhibit a similar formation cycle, usually comprised of initiation of attachment, early irreversible attachment, maturation, followed by detachment and return to a planktonic state *(119)*.

When residing in biofilms, bacteria are believed to be relatively sheltered and protected from the environment *(40)*. In addition, there is a greater availability of nutrients due to the elaborate biofilm architecture, as well as a certain degree of metabolic cooperativity and exchange of genetic material, increasing the chance of survival. As a result, many bacterial species have adapted to life in biofilm consortia *(40)*. One of the first and most studied biofilm formers is *Pseudomonas aeruginosa*, which is frequently associated with recurring infections in cystic fibrosis patients *(38, 57)*. More recently, increasing numbers of studies involving *L. monocytogenes* biofilms have been conducted due to *L. monocytogenes* pathogenic nature, abundance in the environment, and its ability to readily form biofilms *(33, 44, 67)*.

Different species of bacteria are known to have different biofilm architectures and the processes occurring within the biofilm community are relatively complex and coordinated *(40)*. Generally, to form biofilms, bacteria require mechanisms for positioning and attachment, with common ones including flagella, surface translocations, synthesis of cellulose and production of fibrous pellicles, modulation of density, magnetosomes, and aggregation *(40)*. Once the bacteria are attached, formation of monolayers followed by production of extracellular polysaccharides and microcolonies results in maturation of the consortium *(40)*.

1.5.1 *Listeria* spp. biofilms in the food industry

Formation of *Listeria* spp. biofilms in the environment of food processing facilities poses a risk for a product contamination, especially if *L. monocytogenes* cells become detached from a biofilm and reach the final food product. Furthermore, if all the soil and food particles are not removed from a surface and inadequate sanitation takes place, biofilms can continue to expand and result in recurring contamination of food products. Nature of the bacterium, attachment properties and microbial levels can affect the contamination pattern of different surfaces within food processing facilities *(108, 116)*. It has been confirmed in a number of studies that *L. monocytogenes* strains differ in their ability to attach and form biofilms on different types of surfaces *(55, 101, 104, 116, 140)*. Different attachment properties and biofilm formation have been observed among the same *L. monocytogenes* serogroups *(18, 43, 55, 116)*. Folsom et al. *(55)* and Borucki et al. *(18)* reported that the serogroup 1/2 of *L. monocytogenes*

produces more biofilm under decreased nutrient levels when compared to the 4b serogroup. Accordingly, serogroup 1/2 of *L. monocytogenes* is most commonly recovered from food processing facilities *(18, 55)*. In contrast, when it comes to adherence properties Norwood and Gilmour *(116)* observed significantly greater adherence of the 4b serotype of *L. monocytogenes* to stainless steel surfaces compared to the 1/2a serotype. Additionally, Mereghetti et al. *(106)* demonstrated that strains of *L. monocytogenes* serogroup 1/2 have higher minimum inhibitory concentrations for QAC-based sanitizers, benzalkonium chloride and cetrimide than the serogroup 4 strains. The discrepancies in biofilm formation of the same *L. monocytogenes* strains when studied under different conditions *(18, 43, 55)* lead to the conclusion that *L. monocytogenes* is highly adaptive, and that some strains can form biofilms under a great variety of environmental conditions *(55)*. Furthermore, it is highly probable that the interstrain differences in adherence properties are due to factors intrinsic to each strain of *L. monocytogenes (116)*.

Although it has been observed that *L. monocytogenes* represents 1% of the total population when grown in multispecies biofilms on stainless steel, incubation at 10°C for 25 days with a competing microflora comprised of *Flavobacterium* sp., *Pseudomonas* sp., and *Bacillus* sp. did not hinder the growth of *L. monocytogenes* markedly *(78)*. The ability of *L. monocytogenes* to survive and even grow in a hostile environment, such as at refrigeration temperatures and in the presence of competing microflora emphasized the need for increased awareness and more rigorous control measures. The presence of *Listeria*

monocytogenes may often be masked by the competing microflora *(76, 78)*. The nature of the bacterium and the numerous studies investigating the presence of *Listeria* spp. in food processing facilities and in various food products suggest that the incidence of *Listeria* spp., and *L. monocytogenes* in particular, in food processing environments is rather high.

It is difficult to determine whether the presence of the *Listeria* spp. adhered to surfaces in food processing areas is due to specific properties of certain strains or factors that can be controlled by preventative measures and good manufacturing practices. In a study performed by Jessen and Lammert *(79)*, it was shown that removal of biofilms from food processing surfaces cannot be accomplished by a single treatment or a specific detergent and a sanitizer, but rather that physical cleaning was more appropriate for eradication of biofilms. Furthermore, an additional sanitation step prior to swabbing the surfaces only marginally decreases bacterial counts, and does not improve the overall hygienic standard *(79)*.

The formation of microbial communities is a complex occurrence comprised of several different steps. It is understandable that such intricate development requires multiple steps and interventions for biofilms to be eliminated from food processing environments. More importantly, constant monitoring and validation of the cleaning and sanitation protocols is necessary for control of microbial biofilms.

1.6 Susceptibility of *Listeria* spp. to sanitizers

Sanitizers were initially introduced to reduce and/or eliminate certain bacterial populations from areas where these microorganisms were not desirable. When persistent microorganisms were discovered in spite of cleaning and sanitation, certain issues needed to be addressed *(161)*. Initially, the persistence was attributed to inadequate cleaning and sanitation practices *(41, 73)*. However, the discovery of mutant strains that are able to survive even when exposed to sanitizer concentrations previously thought to be lethal *(41, 73)*, suggested that there was a correlation between biofilms and decreased susceptibility to sanitizers *(117, 122)*. Subsequently, the quest to understand why certain bacteria are more or less resistant to specific sanitizers began. Numerous studies have focused on the testing of different sanitizers against both planktonic and sessile bacteria. However, it is difficult to compare the results of these studies due to the inconsistency of experimental designs. As a result, some studies report resistance to sanitizers while others refute it.

Chavant et al. *(31)* investigated the antimicrobial effects of the common sanitizers (e.g. acetic acid, NaOH, Na_2SO_4, Quaternary ammonium compound (QAC)–based sanitizer, and glyceryl monolaurate) against *L. monocytogenes* grown as planktonic cells or as a biofilm. They observed that alkaline sanitizers were more effective than the other sanitizers in reducing the population of slightly more resistant strains of *L. monocytogenes* grown in a biofilm grown for six hours *(31)*. Similarly, the QAC was bactericidal for more than 98% of cells, but some resistance was observed with biofilms grown for seven days *(31)*. The other

sanitizers were not as efficient in killing the pathogen, and differences in effectiveness against planktonic and sessile cells were not observed *(31)*.

Aase et al. *(3)* observed that 10% of the strains of *L. monocytogenes* that they tested were resistant to benzalkonium chloride (i.e. QAC-based sanitizer). Furthermore, they demonstrated that the strains that were resistant to the sanitizer possessed a proton motive force (pmf)-dependent efflux pump, which allowed them to evade ethidium bromide action as well *(3)*. Heir et al. *(69)* observed higher resistance to QAC among isolates recovered from a meat processing facility when compared to human isolates. Similarly, Earnshaw and Lawrence *(47)*, demonstrated differences in sensitivities to three different sanitizers of individual strains of *L. monocytogenes* obtained from poultry processing facilities. However, overall logarithmic reduction for the genotypes that persisted in the processing environment was comparable to those that occurred on a more sporadic and short-term basis. This is in agreement with results reported by Holah et al. *(74)* who also found no difference in the resistance of sporadic or persistent strains of *L. monocytogenes* and *E. coli* isolated from a variety of food processing facilities. Earnshaw and Lawrence *(47)* proposed that persistence in the food processing environment is not a result of inherent resistance but the enhanced ability to adhere for some strains may play an important role in decreased susceptibility to sanitation procedures *(102, 103, 161)*. These findings are in agreement with data provided by Krysinski et al. *(85)* who evaluated a variety of chemical cleaning and sanitizing compounds to determine their efficacy against *L. monocytogenes* that can adhere to surfaces.

Krysinski et al. *(85)* observed that acidic QAC, chlorine dioxide and peracetic acid were the most effective against attached bacteria, while some of the more common sanitizers used in food processing facilities, such as chlorine, iodophors and neutral QAC, were the least effective. In addition, resistance of adherent cells to sanitizers was found to be dependent on the type of surface studied, with polyester/polyurethane surfaces being the most difficult to clean, followed by polyester alone, and stainless steel *(85)*. Nonetheless, the common theme indicates that cleaning must precede sanitizing in order to effectively eliminate attached cells, regardless of the type of sanitizer used.

1.6.1 Role of biofilms in microbial resistance to sanitizers

Once the bacteria adhere and form biofilms, they become more difficult to remove mechanically from surfaces and they may become resistant to sanitizers *(60, 96, 107)*. The decreased susceptibility of bacteria to sanitizers has also been attributed to phenotypic adaptation, genetic alterations and gene acquisitions *(29)*. Mechanisms known to be involved in antibiotic resistance have been speculated to play a role in sanitizer resistance *(29)*. In addition, cross-resistance and co- resistance of sanitizer-resistant bacteria to antibiotics has been observed in some instances, however, it is still an area that requires extensive research *(29, 60)*. Nonetheless, it has been demonstrated that bacteria in biofilms differ both morphologically and genetically from planktonic cells *(30-32, 60, 82, 136)*. The extent to which these distinct characteristics are important in the survival of bacteria when exposed to sanitizers is still rather unclear.

One of the main reasons that biofilms allow survival of bacteria has been attributed to the tightly packed network of bacteria and exopolysaccharides that create a barrier to antimicrobial substances *(19, 60)*. Furthermore, it is believed that bacteria are more resistant to the uptake of toxic substances when they are starving due to decreased growth rate and altered physiology *(19)*. It has been speculated that in addition to the fact that certain types of surfaces are more prone to harboring bacteria *(14, 102)*, the attachment surface provides further protection by exposing only one side of the microorganism to the sanitizer *(19, 89)*. This theory somewhat explains the reason why bacteria become more susceptible to sanitizers once they leave the biofilm consortium; in fact their susceptibility tends to be equivalent to that of planktonic microorganisms *(56)*.

LeChevallier et al. *(89)* showed that the growth medium, temperature and age of biofilms can affect resistance to sanitizers. Biofilms grown for seven days were more resistant to chlorine than biofilms cultivated under the same conditions for two days *(89)*. Additionally speculations that low-nutrient medium can result in changes to the bacterial cell membrane, which in turn can decrease susceptibility to sanitizers, has been suggested *(89)*.

Many theories have been developed to explain the increased resistance to sanitizers of bacteria in biofilms but there is a lack of evidence that one factor contributes more to resistance than another. It is apparent that the behavior of bacteria in communities and the general properties of biofilms are complex issues. As a result, the approach to the control and removal of undesirable

biofilms, especially in the food processing sector, requires multiple interventions and close monitoring.

1.7 Project objectives

A variety of sampling methods for recovery of bacteria from processing environments have been individually evaluated; however, to our knowledge a direct comparison of the methods using a large number of naturally contaminated samples from food processing environments has not been reported. Even though efficiency is essential, very often time, cost, and ease of handling are important factors when selecting a sampling method. To address these issues, the first objective of the project was to evaluate three sampling methods for the recovery of *Listeria* spp. from the environment of two food processing facilities. Over a period of five months the efficiency of the sampling methods, time and the labor requirements were assessed.

Even if an excellent sampling method is practiced in a food processing establishment, the detection of a pathogen is highly dependent on a method of detection. Traditionally, for the isolation and identification of *Listeria*, conventional culture methods have been most commonly used and consequently are considered to be the most reliable. This is demonstrated with their use in investigations of food outbreaks *(42, 105).* However, culture assays are time consuming and laborious. As a result, the food industry is in need of innovative, user friendly and rapid assays for pathogen detection. Therefore, the second phase of the project was to compare three rapid, commercially available detection methods to the conventional culture method. Environmental samples

from meat processing facilities were collected and analyzed for presence of *Listeria* spp. to assess the sensitivity and specificity of each detection method. Knowledge of the ecology of *Listeria* in the food processing chain is important to allow the industry to address probable sources of contamination for different strains of *Listeria* that may have different food safety implications. In addition, the occurrence and distribution of *Listeria* spp. were assessed in the meat processing facilities.

The third objective of the study focused on persistent and nonpersistent strains of *Listeria* and their behavior when exposed to different concentrations of two sanitizers. Following the environmental sampling strains of *L. monocytogenes* were analyzed, and genetic variability of strains was determined as a separate project. Based on genotyping with pulsed field gel electrophoresis (PFGE) persistent and nonpersistent strains were identified for use in this study. Two persistent and two nonpersistent strains and *L. monocytogenes* ATCC 19115 were grown both in a planktonic phase and as biofilms to observe the differences in their behavior when exposed to different concentrations of two sanitizers used in food processing environments.

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2. EVALUATION OF ENVIRONMENTAL SAMPLING METHODS AND RAPID DETECTION ASSAYS FOR RECOVERY AND IDENTIFICATION OF *LISTERIA* SPP. FROM MEAT PROCESSING FACILITIES

2.1 Introduction

As one of the important contaminants associated with foodborne illness, *L. monocytogenes* can cause deleterious health effects, especially in pregnant women, very young, elderly and immunocompromised individuals *(6)*. Its ubiquitous nature and the ability to survive adverse environmental conditions and persist in food processing areas has enabled *L. monocytogenes* to become a notable trespasser in the food industry. The presence of water and food ingredients in food processing areas substantially increases the chance of *L. monocytogenes* survival and proliferation, allowing the microorganism to inhabit floors, walls, and a variety of processing equipment *(18)*. Contamination of ready-to-eat products with *L. monocytogenes* is of major concern to food processors, consumers and health authorities. As a result of numerous listeriosis outbreaks *(7, 36, 37)*, health organizations throughout the world have introduced plans to enhance the safe production of food *(23)*. Studies *(9, 13, 14)* have demonstrated that the food processing environment is a source of contamination for *L. monocytogenes*. Consequently, Health Canada and the Canadian Food Inspection Agency *(23)* and the USDA/FSIS *(5, 39)* have proposed policies requiring meat and poultry processors to test for the presence of *L. monocytogenes* in the processing environment.

A variety of sampling methods are available for recovery of *Listeria* from foods and the processing environment; however, to our knowledge there are no reports that directly compare the methods for recovery of *Listeria* spp. from the food processing environment. Even though efficiency is important, very often time, cost, and ease of handling are major factors when selecting a sampling method. In addition, testing for pathogenic microorganisms in a food processing environment is highly dependent on the use of sensitive and rapid detection assays. Traditionally, conventional culture methods have been considered the be the "gold standard" for the isolation and identification of *Listeria*; however, culture assays are time consuming and labor intensive. There is a need for innovative, user friendly, and rapid pathogen detection methods. Studies involving evaluation of rapid and molecular technologies *(12)* such as PCR, lateral flow immunoprecipitation and enzyme-linked immunosorbent assays have been reported in the research literature. However, most of the studies *(11, 12, 21)* focused on screening of food products for pathogenic microorganisms rather than environmental samples.

To address these issues, a comparison of three environmental sampling methods: 1) cotton swab (CS), 2) sterile sponge (SS), and 3) composite ply tissue (CT), was done to evaluate the recovery of *Listeria* spp. from the environment of two meat processing facilities. In addition, the specificity and sensitivity of three detection methods: 1) Petrifilm™ method 2) an automated chain reaction method and 3) a lateral flow immunoprecipitation (LFI) assay were compared to a conventional culture method for identification of *Listeria* spp. from

samples collected in a food processing environment. In addition, occurrence and distribution of *Listeria* spp. in meat processing facilities were assessed throughout the sampling period from November 2005 until May 2007.

2.2 Materials and methods

2.2.1 Comparison of environmental sampling methods

2.2.1.1 Sample collection

Two federally inspected meat processing facilities located in Edmonton, AB, Canada, were visited over a period of five months in 2005 and 2006. Facilities were each visited six times at three week intervals until a total of 240 samples were collected for each sampling method. In each facility, five locations in an area where raw meat was processed, and five locations in an area where cooked meat was handled were sampled at two sampling times: after sanitation and prior to processing (ACS) and during or after processing but before sanitation (PRO). Facility A produced products such as sausages, cooked hams, pepperoni, wieners, and bacon. Facility B was involved in the production of frozen entrées containing meat. Samples were collected from a variety of locations (Table 2-1), by wiping adjacent areas (ca. 30 x 30 cm) five times vertically and five times horizontally, with each sampling method.

Sterile cotton tipped applicators (CS; Puritan Medical Products Co. LLC, Guildford, Maine) were wetted immediately prior to obtaining the sample by immersing the CS into sterile plastic-coated glass tubes containing neutralizing buffer (NBF; Difco™, Becton, Dickinson and Company, Sparks, MD). After

collecting the sample, the CS was placed into a plastic-coated glass tube with a tightly closed cap where it was kept on ice or refrigerated for no more than 24 h until analysis.

Table 2-1. Areas sampled for recovery of *Listeria* spp. in two federally inspected meat processing facilities.

Type of Processing Area	Source	
	Facility A	**Facility B**
Raw Food	Drain #1	Drain #1
	Drain #2	Drain #2
	Drain #3[1]	Kitchen stairs
	Table	Table #1
	Conveyor	Conveyor #1
	Water Hose[1]	-
	Grinding Machine	-
Ready – to – Eat (RTE) Food	Drain #1	Drain #1
	Drain #2	Drain #2
	Water Hose[1]	Conveyor #2
	Packaging Machine	Table #2
	Conveyor #1[1]	Sink
	Conveyor #2	-
	Meat Rack	-

[1]Sampling locations added for the evaluation of detection methods.

Sterile Ready to Use Sampling Sponge kits (SS; Solar-Cult, Qualicum Scientific Ltd., Ottawa, ON) were used according to the manufacturer's instructions. Briefly, pre-wetted SS were used directly to wipe the areas of interest, after which they were transferred aseptically to sterile sample bags provided with the kit. Sponges were kept in the bags on ice or refrigerated for no more than 24 h until analysis.

Composite ply tissues (CT; Kimberly-Clark Kimwipes® EX-L, Mississauga, ON; 11.4 cm x 21.3 cm) were folded three times according to the protocol

described by Vorst et al. *(40)*, autoclaved (121°C, 30 min), and aseptically transferred to sterile sample bags (11.4 cm x 22.9 cm; Fisherbrand® , Fisher Scientific, Edmonton, AB). Immediately prior to sample collection, 10 ml of NBF was dispensed into the bag to wet the CT. The wetted tissue was pressed manually to remove the excess liquid, and sterile gloves were used to handle the CT during the wiping process. After obtaining the sample, the CT was kept in the sample bag, on ice or refrigerated for no more than 24 h until analysis.

At each sampling time sterility control samples were prepared for the growth medium alone and a sampling device in the growth medium. Positive controls were prepared for the growth medium inoculated with each *L. innocua* ATCC 33090 (American Type Culture Collection, Manassas, VA) and *L. monocytogenes* ATCC 19115 separately, and the sampling device in the medium inoculated with *L. monocytogenes* ATCC 19115.

2.2.1.2 Isolation of *Listeria* spp.

The samples were analyzed for the presence of *Listeria* spp. according to the ISO 11290-1 two-step enrichment protocol *(4)* illustrated in Figure 2-1. Briefly, 90 ml of Demi-Fraser enrichment broth (DFB; Difco™) was added to bags containing SS and CT, while 9 ml of DFB was added to tubes containing CS; all samples were incubated at 30°C for 24 h. After gentle manual squeezing of the bags (where applicable), a 0.1 ml aliquot of DFB culture was transferred to 10 ml of Fraser Broth (FB; Difco™) and incubated at 35°C for 48 h.

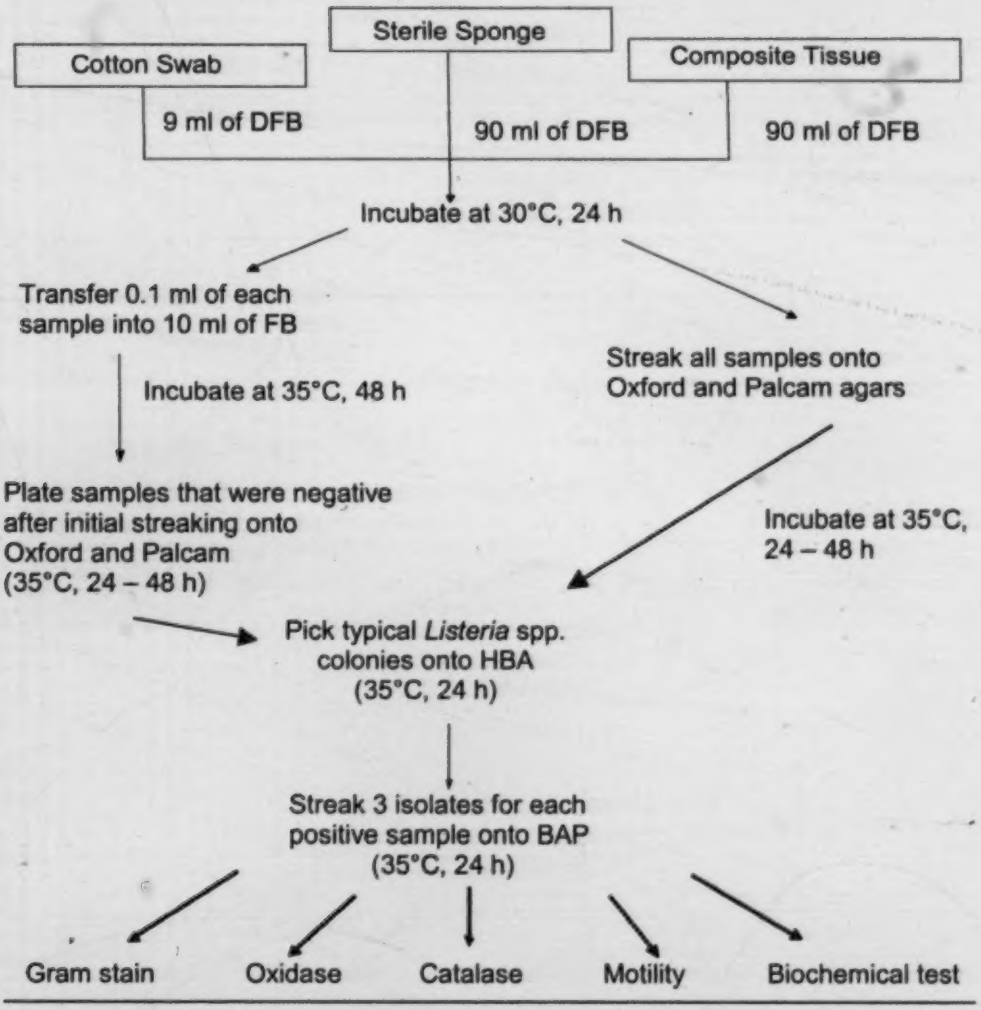
Sterile Sponge
Cotton Swab
Composite Tissue
9 ml of DFB
90 ml of DFB
90 ml of DFB
Incubate at 30°C, 24 h
Transfer 0.1 ml of each sample into 10 ml of FB
Incubate at 35°C, 48 h
Streak all samples onto Oxford and Palcam agars
Plate samples that were negative after initial streaking onto Oxford and Palcam (35°C, 24 – 48 h)
Incubate at 35°C, 24 – 48 h
Pick typical Listeria spp. colonies onto HBA (35°C, 24 h)
Streak 3 isolates for each positive sample onto BAP (35°C, 24 h)
Gram stain
Oxidase
Catalase
Motility
Biochemical test

Figure 2-1. Experimental procedure for recovery of Listeria spp. in samples obtained from the food processing environment, collected with three different sampling methods.

After 24 h of incubation all DFB samples were streaked onto PALCAM (PAL; EMD Chemicals Inc., Gibbstown, NJ) and Oxford (Ox; EMD Chemicals Inc.) agars, and incubated at 35°C for 24 h and 48 h. Screening of presumptive *Listeria* spp. was conducted using Horse Blood Agar (HBA; Difco™) selecting both β-hemolytic and non-hemolytic isolates, after which at least three isolates from one sample were streaked onto defibrinated sheep blood agar (BAP; Oxoid

Ltd., Basingstoke, Hampshire, England) to obtain pure colonies. Further confirmation was based on Gram stain, catalase and oxidase reactions, and motility (MTM; Difco™) at room temperature. Biochemical test strips (Microgen® Listeria ID, Microgen Bioproducts Ltd., Camberley, Surrey, U.K.) were used to differentiate *Listeria* spp.

2.2.1.3 Statistical analysis

Data were entered into spreadsheets and probability values were obtained using McNemar's Chi-square test ($p < 0.05$), calculated with data analysis and statistical software, Stata® (StataCorp LP, College Station, Texas).

2.2.2 Comparison of detection assays

2.2.2.1 Sample collection

Over a period of fifteen months, from March 2006 until May 2007, samples were collected from two federally inspected meat processing facilities in areas where raw and cooked foods were handled, after cleaning and sanitation but prior to processing (ACS), and during or after processing but prior to sanitation (PRO). Based on the findings from the first part of the project, sterile Ready to Use Sampling Sponge kits (Solar-Cult, Qualicum Scientific Ltd.) were used to collect the samples, as described previously. Sampling occurred every three weeks, until a minimum of 100 positive and 100 negative samples per detection method were collected. After 20 sampling times, collection of samples from facility B was stopped due to absence of positive samples. To maximize the

recovery of potentially positive *Listeria* spp. samples, two additional sampling locations were added in the area where raw meat was processed, and two additional locations were included in the area where ready-to-eat products were handled in facility A. Facility A was visited a total of 50 times.

2.2.2.2 Isolation of *Listeria* spp.

To use one sample for all four analyses, 5 ml of Buffered Peptone Water (BPW; Difco™) was added to SS upon receipt in the laboratory. The sample was incubated for 1 h at room temperature (23 ± 2°C) to aid in recovery of any injured cells. After recovery, 3 ml of the sample solution was removed and added to Petrifilm™ Environmental *Listeria* Plates (EL Petrifilm™, 3M™ Microbiology, London, ON). Ninety milliliters of DFB was added to the remaining samples and incubated at 30°C for 24 h. The sample was subsequently tested using the ISO 11290-1culture method *(4)*, the PCR (BAX®, DuPont Qualicon, Wilmington, DE) and the LFI (Reveal® *Listeria* Test System, Neogen Corporation, Lansing, Mich.) methods. At each sampling time, sterility control samples were prepared for the growth medium alone and a sampling device in the growth medium. Positive controls were prepared for the growth medium inoculated with each *L. innocua* ATCC 33090 and *L. monocytogenes* ATCC 19115 separately, and the sampling device in the medium inoculated with *L. monocytogenes* ATCC 19115.

The EL Petrifilm™ method tests were performed according to the Health Canada MFLP-11 method *(1)*.

The ISO 11290-1 *(4)* culture method was performed as previously described.

The automated PCR assays were performed according to the Health Canada MFLP-15 method *(41)* with slight modifications. Briefly, following incubation of DFB at 30°C for 24 h, 0.1 ml was transferred to *Listeria* Enrichment Broth (Difco™) with MOPS (Sigma-Aldrich Inc., St. Louis, MO) added as a supplement and incubated at 35°C for 24 h. Testing was performed using Genus *Listeria* and where applicable, *L. monocytogenes* BAX® System PCR Assay for Screening (DuPont Qualicon, Wilmington, DE) kits. Testing was performed using a BAX® System–PCR Assay with Automated Detection (model QTC-1200 with 1.85 software version; DuPont Qualicon).

Lateral flow immunoprecipitation assays (REVEAL® *Listeria* Test System (Neogen Corporation, Lansing, Mich.) were performed following the manufacturer's protocol with slight modifications. Briefly, 0.1 ml of the DFB sample suspension was transferred to Buffered *Listeria* Enrichment Broth (BLEB; EM Science, EM Industries Inc.) and incubated at 30°C for 21 – 24 h. Following incubation, 2 ml were transferred into a glass tube (16x125 mm) and heated in a water bath at 80°C for 20 min; cooled to room temperature and 135 µl of the cell suspension was transferred to the sample window of the test device. After 20 min results were recorded.

2.2.2.3 Recovery of *Listeria* spp. from EL Petrifilm™, BLEB, and MLEB.

The presence of *Listeria* spp. in samples that tested positive by the three rapid detection methods were confirmed by culturing the samples. After 28 ± 2 h incubation of EL Petrifilm™ plates, a maximum of five presumptive *Listeria* colonies were aseptically transferred with sterile picks from the EL Petrifilm™ into

FB and incubated at 35°C for 48 h. All of the FB samples were plated onto PAL and OX agars and incubated at 35°C for 24 and 48 h.

Similarly, all BLEB and MLEB samples were plated onto PAL and OX agars, and incubated at 35°C for 24 and 48 h. Confirmation of the samples positive for *Listeria* spp. was obtained by plating presumptive *Listeria* spp. colonies onto HBA and BAP agars. Colonies of *Listeria* were confirmed by Gram stain, oxidase, catalase, and motility tests.

2.2.2.4 Statistical analysis

Data were entered into spreadsheets and statistics were generated using Stata® software *(17)*. Culture results were assumed to be free from error, and the estimates of sensitivity and specificity for the EL Petrifilm™, LFI and PCR methods were calculated relative to the results obtained with the culture method.

2.3 Results

2.3.1 Recovery of *Listeria* spp. using different environmental sampling methods.

Over a period of five months 720 environmental samples were collected from meat processing facilities and analyzed for presence of *Listeria* spp. With each sampling method there was fairly equal distribution of the species of *Listeria* that were differentiated using the Microgen® Listeria ID test (Figure 2-2). Of the 240 samples analyzed for each method, the total number of *Listeria* spp. isolated using the SS or the CT was higher ($p<0.01$) than that obtained using the CS (Figure 2-2). There was no statistical difference (Table 2-2) between the number

of positive samples recovered using the SS in comparison to those recovered using the CT swabbing method.

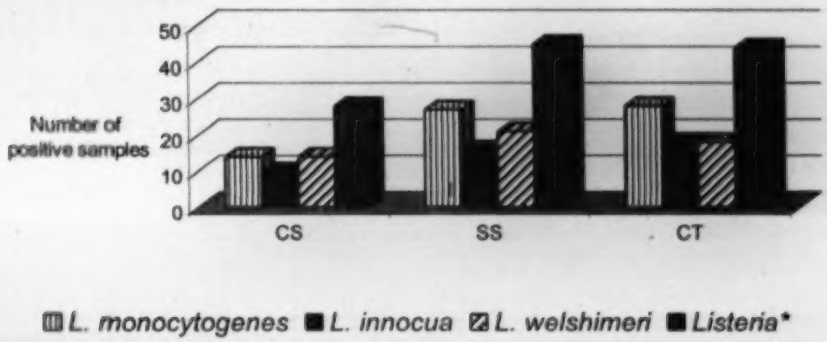

Figure 2-2. Number of samples that tested positive for presence of *L. monocytogenes*, *L. innocua*, *L. welshimeri* and *Listeria* spp. obtained using a cotton swab (CS), a sterile sponge (SS) and a composite tissue (CT) sampling method. Samples (n=720) were collected from the processing environment of two federally inspected meat manufacturing facilities. *Represents the total number of times samples were positive for all species of *Listeria*.

Table 2-2. Probability values obtained using McNemar's Chi-square test for comparison of three environmental sampling methods for recovery of *Listeria* spp. from meat processing environment[1].

	Cotton Swab	Sterile Sponge	Composite tissue
Cotton Swab	-	0.0025	0.0015
Sterile Sponge	0.0025	-	1.0000
Composite Tissue	0.0015	1.0000	-

[1] Methods are significantly different if $p<0.05$

2.3.2 Evaluation of detection assays for *Listeria* spp.

Samples were collected until a minimum of 100 positive and 100 negative samples were obtained for each detection method. The requirement of 100 positive and 100 negative samples for the PCR and LFI methods was reached after 11 sampling times; however, for the EL Petrifilm™ method, the requirement

was reached after 14 sampling times, which resulted in a greater number of samples tested.

After 11 sampling times, a total number of samples collected was 328. The culture method identified 110 samples that were positive for *Listeria* spp. while the PCR and LFI assays identified 109 and 105 positive samples, respectively. Following the 14th sampling, the total number of samples collected and analyzed with the culture assay and the EL Petrifilm™ method was 440. From 440 samples tested, 110 were identified as positive for *Listeria* spp. with the conventional culture method. The EL Petrifilm™ method recognized 103 positive samples; however, out of these samples 24 were false positive. Out of the 337 EL Petrifilm™ samples that were negative for *Listeria* spp. 77 samples were identified as false negative.

When colonies were isolated from the 103 presumptive positive EL Petrifilm™ samples, only 37.9% of these samples were confirmed positive for *Listeria* spp. by the culture method. The diversity of the colonies grown on the EL Petrifilm™ can be seen in Figures 2-3 to 2-5. All colonies that were circled were similar in color but with different intensities of pink; however, the bright pink color of colonies was not necessarily an indication of *Listeria* spp. Culturing of the presumptive *Listeria* spp. colonies revealed several false positive results.

Sensitivity, specificity and kappa values for the three detection methods for identification of *Listeria* spp. in environmental samples using the culture method as the "gold standard", are shown in Table 2-3. LFI and PCR were highly sensitive and specific when compared to the culture method.

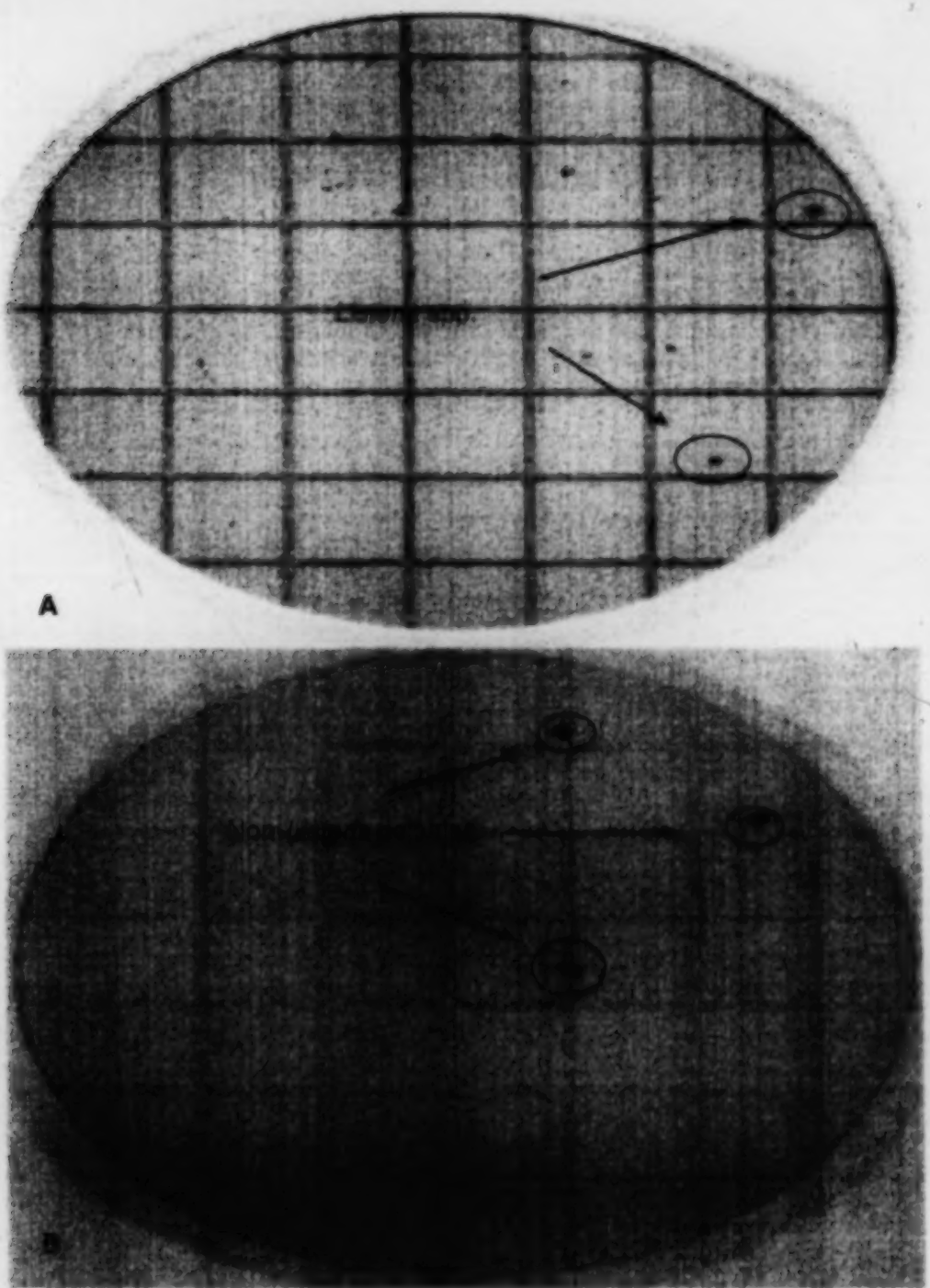

Figure 2-3. Photographs of EL Petrifilm™ plates after 28 h incubation at 35°C, of samples recovered from a conveyor (A) and a hose (B) located in the area where raw meat was processed in facility A. Colonies that are circled in B indicate those which were not confirmed as *Listeria* spp.

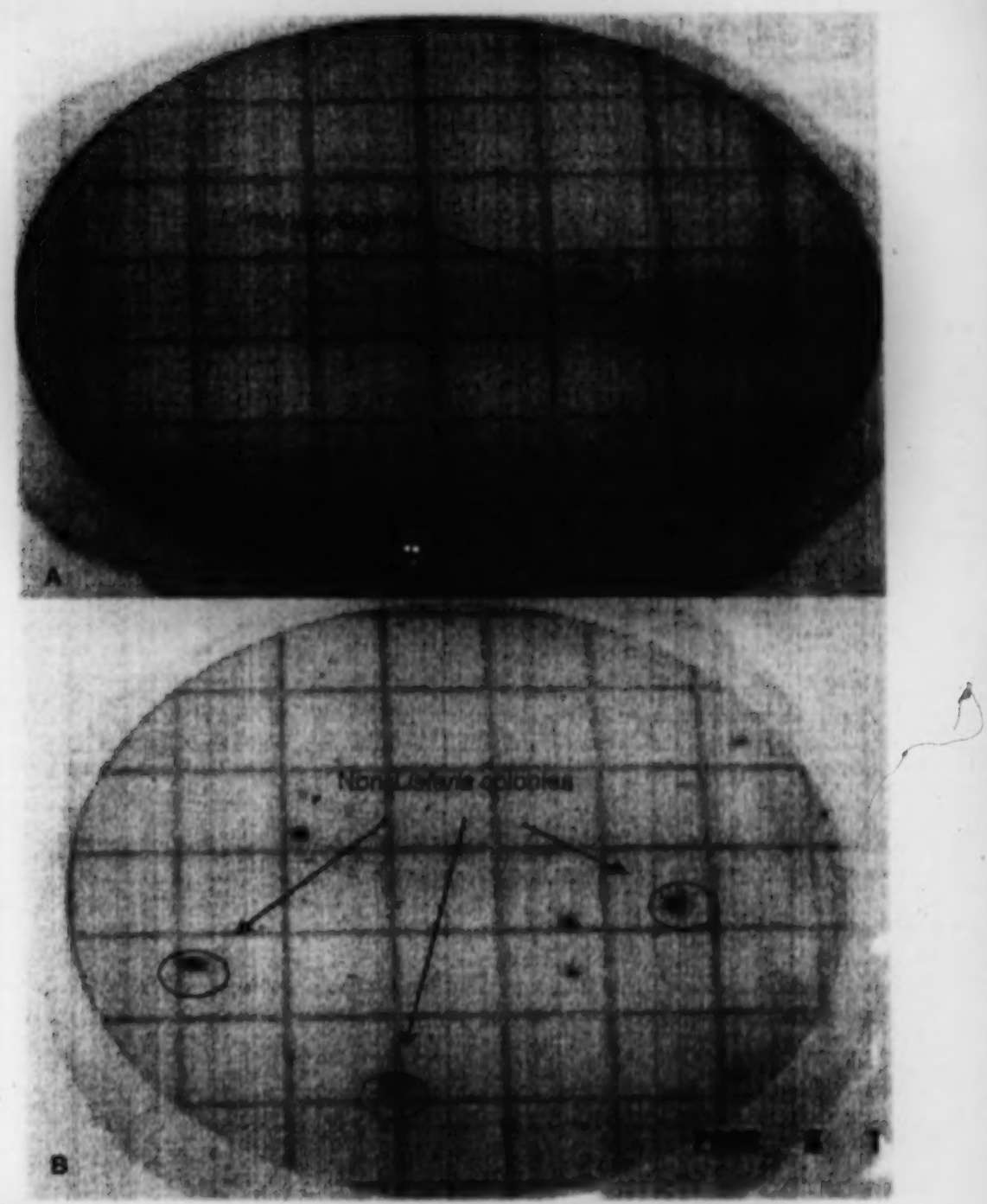

Figure 2-4. Photographs of EL Petrifilm™ plates after 28 h incubation at 35°C, of *L. monocytogenes* ATCC 19115 (A) and a sample recovered from kitchen stairs located in the area where raw meat was processed in facility B (B). Colonies that are circled in B indicate those that were not confirmed as *Listeria* spp.

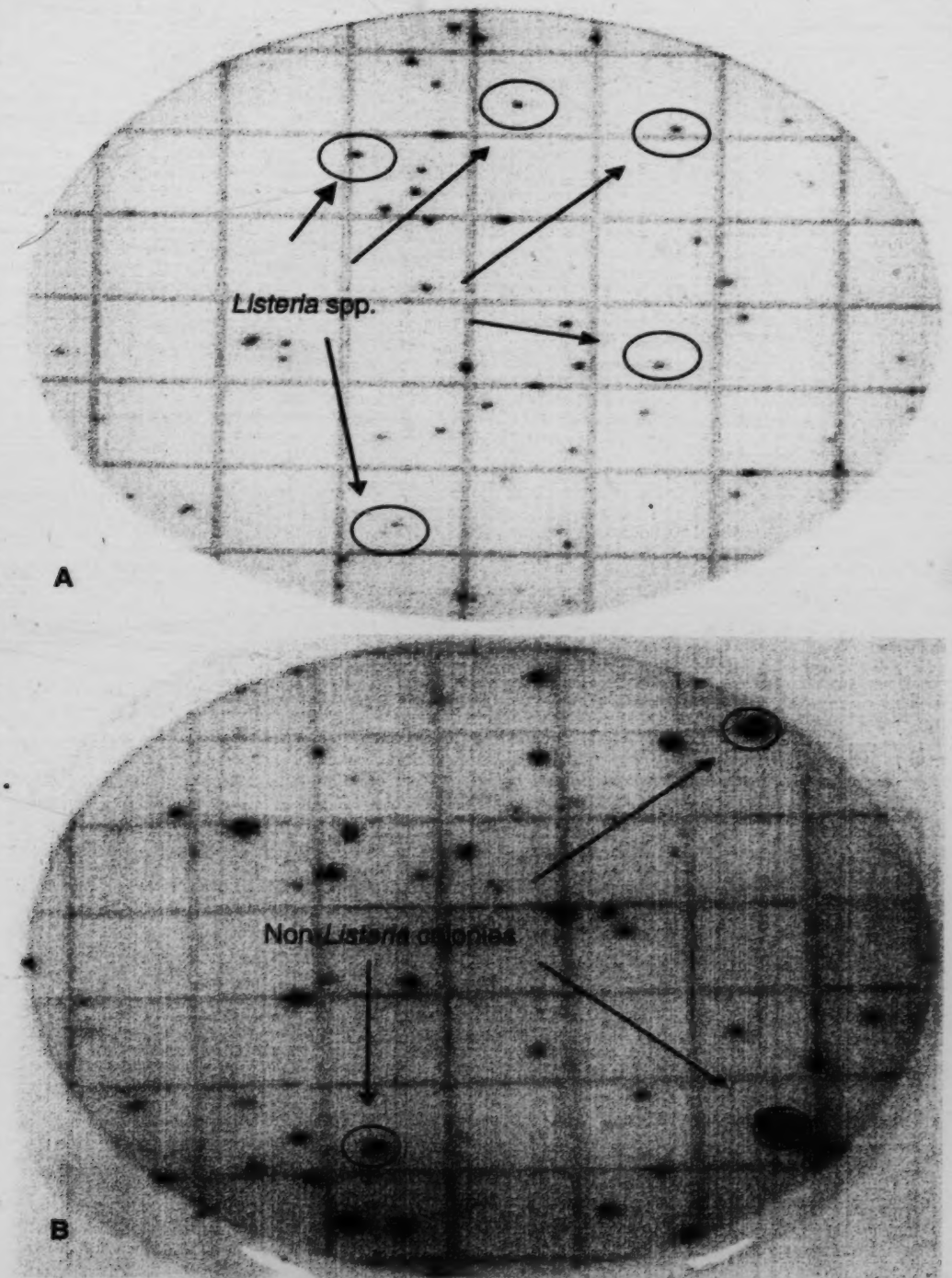

Figure 2-5. Photographs of EL Petrifilm™ plates after 28 h incubation at 35°C, of samples recovered from the drain #2 (A) in the area where cooked products were handled, and a table (B) located in the area where raw meat was processed in facility A. Colonies that are circled in B indicate those that were not confirmed as *Listeria* spp.

Based on kappa values, there was excellent agreement of both the PCR and LFI methods with the culture method, while moderate agreement was observed for the EL Petrifilm™ method. Overall, EL Petrifilm™ plates were less efficient in the detection of *Listeria* spp. from environmental samples in comparison to the other methods tested.

Table 2-3. Sensitivity, specificity, and kappa values of the EL Petrifilm™, LFI and PCR methods relative to the culture method for identification of *Listeria* spp. in samples obtained from the environment of two federally inspected meat processing facilities.

	EL Petrifilm™	Lateral flow immunoprecipitation	PCR
Sensitivity (%) (95% CI)	50.6 (42.5 – 58.7)	95.5 (89.7 – 98.5)	99.1 (95 – 100)
Specificity (%) (95% CI)	91.5 (87.7 – 94.5)	100 (98.3 – 100)	100 (98.3 – 100)
Kappa (95% CI)	0.457 (0.370 – 0.544)	0.965 (0.935 – 0.995)	0.993 (0.980 – 1.00)

2.3.3 Occurrence and distribution of *Listeria* spp. in the facilities

Over a period of 19 months, 820 environmental samples were collected from areas in which raw or cooked meat products were handled in two federally inspected meat processing facilities. From the 820 samples analyzed, 249 were positive for *Listeria* spp. The total number of samples collected in facility A was 652, while 168 samples were collected in facility B. Overall, 248 positive samples for *Listeria* spp. were recovered from facility A, and one positive sample was obtained from facility B in the area where raw meat was processed.

A large proportion of the samples (89.4%) that were positive for *Listeria* spp. were collected during or after processing (PRO) in the area where raw meat was processed (Table 2-4). Similarly, after cleaning and sanitation but prior to

processing (ACS) more positive samples were collected in areas where raw meat was processed (81.2%) than in the environment where cooked products were handled.

Table 2-4. The number of samples collected from two meat processing facilities either after cleaning and sanitation (ACS) or during or after processing (PRO) from either an area where raw meat was processed or an area where cooked products were handled that tested positive for *Listeria* spp.

	No. of positive samples/total number collected			
	ACS		PRO	
	Raw Product Area	Cooked Product Area	Raw Product Area	Cooked Product Area
Facility A (n=652)	95/163	22/163	117/163	14/163
Facility B (n=168)	0/42	0/42	1/42	0/42
Total	95/205	22/205	118/205	14/205

However, it is interesting to note that in the area where cooked products were handled, a slightly higher number of samples that were positive for *Listeria* spp. were collected after cleaning and sanitation (10.7%) than during or after processing (6.8%; Table 2-4).

From the 248 positive samples recovered from facility A, 212 samples were acquired in the areas where raw meat was processed, and 36 were obtained in the areas where cooked meats were handled. Differentiation of the species of *Listeria* for the positive samples obtained during 19-month sampling period for both ACS and PRO sampling times is presented in Figure 3-6. More samples that tested positive for *Listeria* spp. were obtained during or after processing (PRO) than after cleaning and sanitation (ACS). Distribution of the samples that tested positive for *L. monocytogenes* was the same for both the ACS and PRO sampling times. More samples tested positive for *L. innocua* and *L. welshimeri* for the PRO sampling time compared to the ACS (Figure 2-6).

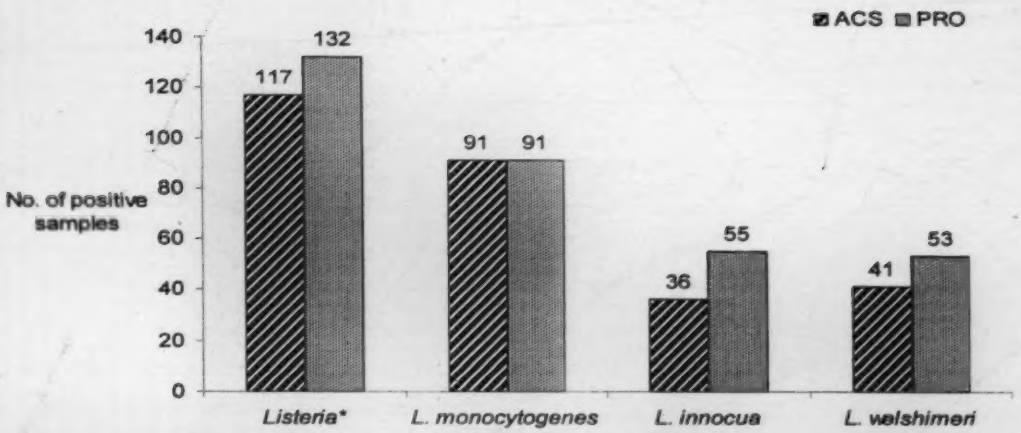

Figure 2-6. The number of samples that tested positive for different species of *Listeria* collected either after cleaning and sanitation (ACS) or during or after processing (PRO), from the areas where raw and cooked products were processed in two meat manufacturing facilities.*Represents the total number of times samples were positive for all species of *Listeria* out of 820 samples collected.

The number of samples obtained from the different sampling locations in facility A, and the distribution of the species of *Listeria* obtained from the different locations is shown in Figure 2-7. Samples that were positive for *Listeria* spp. were recovered 98% of the time in Drain #1, which was located in the area where raw meat was processed in facility A. A high occurrence of contamination was also observed for drains #2 and #3 located in the area where raw food was processed in facility A, from which samples positive for *Listeria* spp. were collected 92% and 70% of the time, respectively (Figure 2-7). *L. monocytogenes* was most frequently isolated from drains #1, #2, and #3, and the grinding machine in the area where raw meat was processed, while in the area where cooked foods were handled, one drain in particular (Drain #2) was more often contaminated than the rest of the locations that were sampled in that area. *Listeria monocytogenes* was isolated more than once from all the locations

sampled in the area where raw meat was processed, and at least once in six out of seven locations swabbed in the area where cooked products were handled. Additionally, *L. monocytogenes* (73.0%) was the species most frequently isolated from the positive samples, followed by *L. welshimeri* (37.8%), and *L. innocua* (36.1%; Figure 2-7).

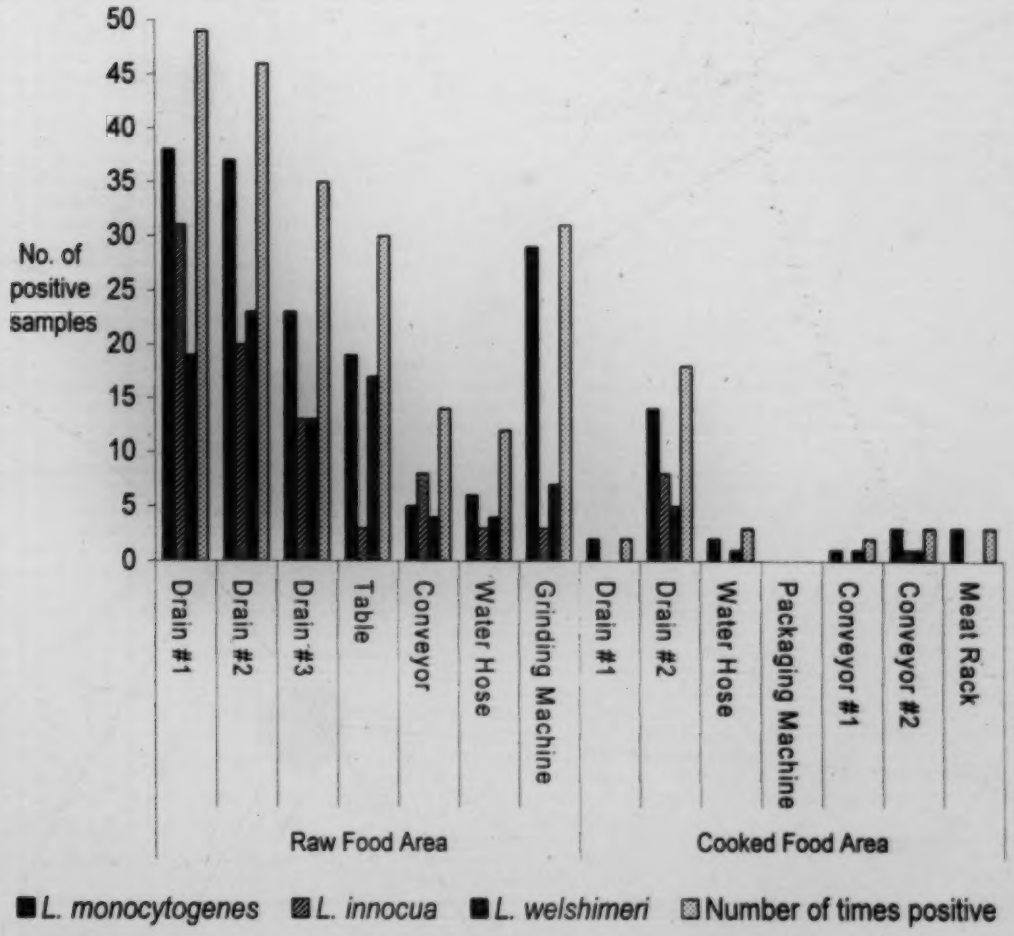

Figure 2-7. The distribution and number of the samples that tested positive for *L. monocytogenes*, *L. innocua*, or *L. welshimeri* and the total number of times samples were positive for *Listeria* spp. Samples (n=820) were obtained from the different sampling locations in facility A.

2.4 Discussion

Although listeriosis is regarded as low risk illness for the majority of the healthy population *(15)*, for immunocompromised individuals the consequences may be fatal. As a result, many countries have a policy of "zero tolerance" when it comes to presence of *L. monocytogenes* in ready-to-eat foods that support the growth of the microorganism and have a shelf life longer than ten days, such as soft cheese, liver pâté, hot dogs, cold smoked rainbow trout and processed deli meats *(2, 3, 15, 23, 26, 36, 38)*. With such strict rules, importance is placed on the appropriate sampling and detection methods, as well as the adequate sanitation practices. However, there is no standardized swabbing protocol for environmental sampling. Materials such as cellulose sponges, composite-ply tissues, and cotton tipped swabs have been used in microbial analyses for beef carcass tissue *(28)* and pork loin primal cuts *(20)*; however, there is a lack of published data that reports efficiency of swabbing methods when used in an environmental setting.

Vorst et al. *(40)* evaluated sterile environmental sponges, sterile cotton-tipped swabs, sterile calcium alginate fiber-tipped swabs, and one-ply composite tissues for the quantitative recovery of *L. monocytogenes* from 304-grade stainless steel plates. They found that the one-ply composite tissue is superior ($P<0.001$) in the quantitative recovery of *L. monocytogenes* in comparison to the environmental sponge, cotton-tipped swab or the calcium alginate fiber-tipped swab. The results of the current study are in accordance with Vorst et al. *(40)* with the composite ply tissues being a suitable method for the recovery of *Listeria*

spp.; however, the current results show that the SS was equivalent to the CT in recovery of *Listeria* spp. Sterile sponges were more appropriate for qualitative sampling performed in the food processing areas due to the ease of handling and good recovery of *Listeria* spp. Results of the current study revealed that the cotton tip swab was the least efficient in the recovery of *Listeria* spp., which is also in agreement with the results of Vorst et al. *(40)*.

It is important to consider the extent to which the studies can be compared, as they were performed under very different conditions. Vorst et al. *(40)* simulated bacterial behavior in food processing plants using stainless steel coupons inoculated with pure cultures. Food production areas are often inhabited with a variety of microorganisms and thus the outcome of studies in the processing environment may be very different from laboratory studies intended to simulate a processing environment. The conditions of the surface and the state of the microflora may affect the numbers of bacteria recovered by any sampling method *(20, 31)*. Also, when choosing a sampling method, the type of the food being processed, potential microflora present, material of the sampling device used, as well as whether qualitative or quantitative assays are required, need to be considered. Vorst et al. *(40)* evaluated quantitative recovery of *Listeria* while we were interested in a qualitative method since current policies are based on the presence or absence of *Listeria* in ready-to-eat foods and processing environments.

In addition, ease of handling and the cost of the sampling device are important factors that cannot be overlooked. While CT is less expensive and

comparable to SS when it comes to recovery of *Listeria* spp., the time it takes to prepare CT (ca. 65 min for 40 samples) and a greater chance of contamination during preparation steps are considerable disadvantages of the CT swabbing method. Although the methods were similar in their ability to recover *Listeria* spp., the time and labor in the preparation and difficulties in handling the CT for environmental sampling may make it undesirable for routine sampling in food processing environments.

Testing food products for the presence of pathogenic microorganisms is generally a priority; however, for pathogens such as *L. monocytogenes* that can persist in food manufacturing facilities for a long time and result in recurring product contamination *(18)*, testing of the processing environment is critical. To minimize labor and time associated with conventional culture methods, a number of user-friendly and rapid pathogen detection methods have been developed. A variety of factors can affect the performance of detection methods *(10)* and for this reason many of them have been developed to accommodate specific types of foods, as well as microorganisms of interest. For instance, commercially available LFI and ready-to-use PCR kits are available for testing of a wide variety of foodborne pathogens, including *Salmonella, Campylobacter, Escherichia coli* O157:H7, and *Listeria* spp. In this study, the detection of *Listeria* spp. by three rapid technologies was compared with a standard culture method.

Conventional culture assays were historically regarded as the most reliable methods *(16, 29)*; however, because they are time-consuming, the food industry, especially manufacturers of perishable foods, need more rapid detection

alternatives. PCR based technologies, such as the BAX® system, have reduced the time required for detection of *Listeria* spp. to 48-50 h after sampling *(41)*. In the current study BAX® was an excellent alternative to the culture method, with high sensitivity (99.1%) and specificity (100%) for detecting *Listeria* spp. in environmental samples. Similarly, Hoffman and Weidmann *(24)* in 2001 reported sensitivity and specificity rates of 94.7% and 97.4% when they followed the manufacturer's protocol. Norton et al. *(32)* also evaluated performance of BAX® for the detection of *Listeria* spp. in cold-smoked fish products and environmental samples. The sensitivity (89.9%) and specificity (96.2%) values they obtained were lower than the results in the current study; however, modifications of the methodology may explain some of the differences. In this study, a two-step enrichment protocol was used. In contrast, Norton et al. *(32)* used a single-step enrichment in *Listeria* enrichment broth (LEB), which may have resulted in low numbers of *Listeria* in the enrichment culture, and this may have impacted the sensitivity of the BAX® system *(32)*. In addition, since 2000 when Norton et al. *(32)* published their findings, improvements of the BAX® system through the inclusion of an internal positive control in each reaction tube has been implemented to confirm that substances present in the sample do not inhibit the PCR reactions *(24)*. Other studies comparing PCR-based methods, such as BAX®, to culture assays for detection of *L. monocytogenes* reported sensitivity and specificity rates of 92.7% and 84.9%, respectively, for samples obtained by swabbing the equipment in a chicken nugget processing plant *(33)*, and values of 84.8% and 100% for sensitivity and specificity, respectively, when testing raw fish

materials *(24)*. Becker et al. *(11)* noted a sensitivity of 100% and a specificity of 92% in a study evaluating *L. monocytogenes* detection in cold smoked vacuum packaged salmon, with the results being similar to those obtained in the present study. Although sensitivity and specificity values seem to vary between studies, based on the published data and results obtained in the current research, the BAX® system for screening *Listeria* spp., and *L. monocytogenes* in particular can be effective means of monitoring the bacteria in smoked fish *(32)* and meat processing environments *(33)*.

The lateral flow immunoprecipitation device was slightly lower in sensitivity (94.5%) compared to culture than the BAX® system for detection of *Listeria* spp. in environmental samples; however, the specificity rate (100%) was high indicating the capability of the method for identification of negative samples efficiently. Bohaychuk et al. *(12)* also reported specificity of 100% for LFI when screening for *Listeria* in meat and poultry products. Rodrigues et al. *(33)* evaluated the *Listeria* Rapid Test-Clearview™, which is based on use of the specific monoclonal antibodies against flagellar antigen B of *Listeria*, and in principle it is similar to the LFI device tested in the current study. Sensitivity and specificity values of 98.5% and 100%, respectively, for Clearview™ *(33)* were reported, which are comparable to the current results for LFI, indicating that lateral flow immunoprecipitation based assays are excellent alternatives to conventional culture methods.

Unlike the PCR and LFI methods, the EL Petrifilm™ method demonstrated poor performance in its ability to detect *Listeria* spp. in environmental samples

collected from meat processing facilities. Although sensitivity and specificity values of 98% and 100%, respectively, have been reported by 3M Microbiology *(8)*, in the current study sensitivity and specificity values of 50.6% and 91.5% were obtained. Groves and Donelly *(22)* found sensitivity and specificity rates of 87.5% and 96.7%, respectively, for the EL Petrifilm™ method used to detect *Listeria* spp. on environmental and food contact surfaces in a dairy plant. These results along with the results of the current research indicate that the EL Petrifilm™ may be used for routine screening of the food processing facilities due to the fairly low number of false positive samples reported. However, of the three rapid technologies tested, the EL Petrifilm™ was the least efficient in detection of *Listeria* spp. in the food manufacturing environment as a result of the high number of false negative samples. In addition, a large proportion of the colonies growing on the EL Petrifilm™ that appeared to meet the criteria set by 3M™ Microbiology for identification as *Listeria* spp. were not able to be confirmed as *Listeria* spp. Poor detection of *Listeria* spp. in the environmental samples by the EL Petrifilm™ might be attributed to low numbers of the microorganisms present, or due to overgrowth of competitive microflora (Figures 2-3 to 2-5). In addition, comparison of the studies done on naturally contaminated samples, such as those obtained from the food processing facilities is limited due to the differences in sampling procedures, food matrices, as well as testing conditions. Therefore, the results obtained in the study and data presented in other studies have to be evaluated individually.

The overall occurrence and distribution of *Listeria* spp. in meat manufacturing environments were evaluated from November 2005 until May 2007. *Listeria* spp., particularly, *L. monocytogenes* were found in areas where raw meat was processed and in areas where cooked products were handled in facility A, which processes a variety of meat products. No samples obtained from facility B contained *L. monocytogenes*.

The overall contamination of environmental samples with *Listeria* spp. from facility A was 38.0% which is comparable to the contamination of samples from environment of a poultry processing plant (37%) reported by Lawrence and Gilmour *(27)*, but it is lower than the 59% observed by El-Shenawy *(18)* in ice-cream processing facilities. Chasseignaux et al. *(13)* observed 38.9% sample contamination from the environment of poultry processing facilities and 37% in pork processing environments, which are very close to the results obtained in the current study. However, in comparison to the fish industry, the contamination observed in facility A is considerably higher than the 7.2% *(30)* and 11.8 % *(25)* positive samples recovered from fish processing factories, and 20% and 29% contamination of samples with *Listeria* spp. from a fish slaughterhouse and smokehouse, respectively, reported by Rørvik et al. *(34)*.

The occurrence of *L. monocytogenes* in environmental samples collected during the 19-month sampling period was 22.2%. Results from the current study are considerably lower than the 55% contamination found in samples from delicatessen meat processing facilities in France *(35)*. Gianfranceschi et al. *(19)* observed that only 27.9% of samples from the environment and work surfaces

were contaminated with *L. monocytogenes* in three different food processing facilities in a study carried out in Italy between 1990 and 1999. In particular, 9.7% of samples obtained from meat production areas were contaminated with *L. monocytogenes (19)* which is considerably lower than the occurrence observed in the current study (22.2%). The results from the current research indicated that 51.9% of samples collected in the areas where raw meat was processed were positive for *Listeria* spp., which is considerably higher than the 26% reported in the environment of a poultry plant in the area where raw meat is handled during a six months study conducted by Lawrence and Gilmour *(27)*. However, only 8.8% of the samples recovered from the area where cooked products were handled contained *Listeria* spp., which is notably lower than 15% samples that were positive for *Listeria* in areas where raw and cooked poultry is handled *(27)*. In the current research, the occurrence of contamination of the food production areas after cleaning and sanitation was lower than that observed during or after processing, which is in accordance with that reported for the contamination of the environment and products produced in dried sausage processing facilities *(38)*, delicatessen meat processing facilities *(35)* and fish slaughter- and smokehouses *(42)*. The number of samples that tested positive for *L. monocytogenes* was the same for both sampling times, after cleaning and sanitation, and during or after processing, while more *L. innocua* and *L. welshimeri* were recovered from samples obtained during or after processing than after cleaning and sanitation (Figure 2-6). In the current study a slightly higher number of samples that tested positive for *Listeria* spp. were obtained after cleaning and sanitation in the areas

where cooked products were handled, indicating that these areas may harbor persistent strains that are possibly resistant to sanitizers and more difficult to eliminate *(42)*. In contrast, Salvat et al. *(35)* reported that samples obtained from areas where RTE products are handled were less likely to be contaminated with *Listeria* spp.

In facility B, only one sample tested positive for *Listeria* spp., in particular *L. innocua*, indicating very effective cleaning and sanitation regimes. Even though it is hard to eliminate all *Listeria* spp. from the food processing environment, this study shows that with good cleaning and sanitation practices it is possible to achieve low numbers of *Listeria* spp. However, it is important to note that the two facilities visited in this project process different meat products and their processing protocols are notably different. In addition, facility A is a larger scale facility in comparison to facility B. Therefore, the extent to which the contamination of the two processing facilities can be compared is limited. Nevertheless, the variability in contamination of the two facilities further suggests that establishing contamination patterns for *Listeria* spp. in the food processing environment can be particularly difficult. If a high occurrence of contamination is observed in any food processing facility, the cleaning and sanitation regimes need to be improved, with a focus on the critical control points, which in most facilities are different.

Results of the current study indicated that there was an excellent agreement of the LFI and PCR methods with the culture method, while moderate agreement was observed for the EL Petrifilm™ method. Overall, the EL

Petrifilm™ was found to be easy to use but less efficient in detection of *Listeria* spp. in environmental samples, while the LFI and BAX® methods were found to be excellent alternatives to the culture method, considering performance, time and labor inputs.

In addition, the occurrence of contamination with *Listeria* spp. of the meat processing facilities in Edmonton varies tremendously from one facility to the other. Further investigation is required to determine reasons for higher contamination of facility A in comparison to facility B, particularly focusing on the differences in sanitation protocols, equipment and facility design, as well as management and personnel attitude and awareness regarding the presence of *Listeria* spp. Even though there have not been any documented outbreaks associated with *L. monocytogenes* in meat from the two facilities involved in the study, the presence of *L. monocytogenes* in the areas where cooked products are handled is a serious concern. When it comes to *L. monocytogenes*, the importance of adequate cleaning, good manufacturing practices, and education of the facility personnel cannot be underestimated. Therefore, in addition to regular food sampling to assess food safety and quality, it is critical to evaluate cleaning and sanitation protocols on a regular basis.

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3. SUSCEPTIBILITY TO SANITIZERS OF PERSISTENT AND NONPERSISTENT STRAINS OF *L. MONOCYTOGENES* GROWN IN PLANKTONIC AND BIOFILM STATES

3.1 Introduction

L. monocytogenes has been shown to persist on a variety of surfaces in food processing facilities *(9, 18)*. Different hypotheses have been proposed for persistence in food processing areas, some of which include inadequate cleaning and sanitation practices *(2)*, particular food environments and surfaces being more prone to harboring pathogenic microorganisms *(18, 26, 27)*, formation of biofilms *(13)*, increased resistance to certain sanitizers *(13, 16)*, and variability of *L. monocytogenes* strains in their ability to form biofilms and adapt to environmental stressors *(6, 19)*. Although the underlying cause of persistence is unknown, the ability of these organisms to persist on both environmental and equipment surfaces is a concern for the food industry.

The type of surface and the ability to form biofilms can affect the susceptibility of *L. monocytogenes* to different sanitizers *(13, 24, 27)*. Stainless steel surfaces allow more biofilm formation in comparison to plastic *(13)* and buna-n rubber (rubber containing acrylonitrile butadiene, which is commonly used in food processing equipment) surfaces *(27)*. However, more successful inactivation or removal of cells within biofilms is observed on stainless steel surfaces as opposed to plastic *(13)* and rubber surfaces *(24, 27)*. Studies have demonstrated that commonly used sanitizers such as chlorine, iodophors and certain quaternary ammonium compounds (QAC) at concentrations

recommended by manufacturers may not be effective in the inactivation of adherent cells of *L. monocytogenes (13, 21)*. Most sanitizers can effectively reduce the numbers of or inactivate planktonic cells of *L. monocytogenes (13)*; however, when *L. monocytogenes* are grown in biofilms, higher concentrations of sanitizers are required to have a bactericidal effect *(7, 13)*. Lundén et al. *(17)* demonstrated that strains of *L. monocytogenes* that can persist in the processing environment for long periods of time exhibit enhanced adhesion to stainless steel surfaces after a short contact time compared to nonpersistent strains; however, if these strains were observed after a longer contact time the differences were marginal. Even if certain strains of *L. monocytogenes* adhere better to surfaces, it is not necessarily an indication that they are better biofilm formers than those strains that do not exhibit strong adherence properties *(7, 10)*. Scanning electron microscopy analysis of various persistent and nonpersistent strains did reveal remarkable differences in the biofilm structures of persistent and nonpersistent strains *(4)*. The behavior of organisms on surfaces is a complex process affected by many factors.

Contradictory results have been reported regarding the resistance to sanitizers of persistent strains of *L. monocytogenes* when compared to nonpersistent strains when cells are grown in planktonic state and in biofilms. Inter-strain variation in the resistance of planktonic cells of individual strains of *L. monocytogenes* to low concentrations of different sanitizers has been observed *(1, 7, 19)*; however, correlation between resistance to sanitizers and the ability of an organism to persist in the environment when tested in a suspension has not

been reported *(7, 11, 12)*. It is important to note that resistance of *L. monocytogenes* to concentrations of sanitizer equal to or higher than those recommended by the manufacturer when bacteria were grown in a liquid suspension has not been established.

Similarly, research on resistance to sanitizers when bacteria are attached to various surfaces is somewhat contradictory. Krysinski et al. *(14)* demonstrated that a variety of biocides, such as neutral and acidic QAC sanitizers, mixed halogen, acid anionic, and peracetic acid based sanitizers were effective in inactivation of cells of *L. monocytogenes* adhered to stainless steel even when concentrations recommended by manufacturer were applied. In contrast, Frank and Koffi *(8)* demonstrated that some strains of *L. monocytogenes* grown on glass slides have the ability to develop resistance to quaternary ammonium and anionic acid sanitizers under different growth conditions.

Differences in experimental design, such as application of different assays to test for resistance to sanitizers and the lack of standardization for sanitizer concentrations that classify bacteria either resistant or susceptible make comparison of studies particularly difficult. It is probable that resistance of a bacterial strain to specific sanitizers depends on the history of the bacterium, including source of the strain, inherent and acquired traits, previous exposure to different sanitizers as well as different concentrations of sanitizers.

The aim of this study was to investigate the susceptibility of strains of *L. monocytogenes* acquired from meat processing environments to two sanitizers used by the meat processing establishment. The strains were grown in liquid

suspension and attached to stainless steel or plastic surfaces. A further objective was to determine if persistent strains of *L. monocytogenes* were more resistant to these sanitizers compared to nonpersistent strains and a laboratory strain of *L. monocytogenes*.

3.2 Materials and methods

3.2.1 Cultivation of *L. monocytogenes*

Persistent and nonpersistent strains of *L. monocytogenes* were identified using pulsed field gel electrophoresis (PFGE) as part of a concurrent study. Identical strains that were repeatedly recovered from the processing environment were considered persistent, and strains that were recovered sporadically were considered nonpersistent. Five strains of *L. monocytogenes* [two persistent (P1 and P2), two nonpersistent (NP1 and NP2), and *L. monocytogenes* ATCC 19115 (ATCC)] were inoculated separately into 10 ml of tryptic soy broth (TSB; Bacto™, Becton, Dickinson and Company, Sparks, MD) and incubated at 35°C for 18 to 24 h to obtain ca. 10^9 CFU/ml, which was measured using a spectrophotometer (GENESYS™ 20, Thermo Fisher Scientific Inc., Edmonton, AB) set at 600 nm. When necessary, the test suspension was adjusted using TSB to reach an OD_{600} between 0.3 and 0.4 nm, corresponding to ca. 10^9 CFU/ml. Following the adjustment, planktonic cells were centrifuged (1,578 x*g*; Jouan C 3.12 Model, Canberra Packard, Canada), decanted and resuspended in 10 ml of 0.85% saline (Sigma Chemicals Co., St. Louis, MO). The test suspension for the assay with planktonic cells was prepared by diluting serially in 0.85% saline to obtain

ca. 10^7 CFU/ml. The test suspension for use in the biofilm assay was diluted in TSB to obtain ca. 10^5 CFU/ml. Viable cell counts were determined for each strain by serial dilution of the test suspension in sterile 0.1% peptone water (Difco™) and using Tryptic Soy Agar (TSA; Difco™) incubated at 35°C for 24 h to confirm the starting concentration of bacteria used in the experiments.

3.2.2 Preparation of stainless steel coupons

Stainless steel coupons (type 304, SS-8, No. 4 finish, 12 mm diameter; Stanfos, Edmonton, AB, Canada) were soaked overnight in 1 N NaOH, rinsed three times in sterile distilled water and autoclaved at 121°C for 30 min prior to use. For coupons that were used more than once, coupons were soaked overnight in 100% ethanol, followed by manual cleaning of the surface with a soft brush to remove any residual biofilms, after which coupons were placed in 10% bleach and allowed full contact for 1 h. Prior to use the coupons were rinsed with sterile distilled water and autoclaved at 121°C for 30 min.

3.2.3 Preparation of sanitizer

The two sanitizers used in the study were E-San® 10%, a QAC-based sanitizer containing 5% N-alkyl dimethyl benzyl ammonium chloride and 5% N-alkyl dimethyl ethyl benzyl ammonium chloride (Epsilon Chemicals Ltd., Edmonton, AB, Canada), and Perox-E® (Epsilon Chemicals Ltd.) containing hydrogen peroxide and acetic acid as active compounds. Sanitizers were stored at 4 ± 2°C according to the manufacturer's recommendations. For experiments involving sanitizer susceptibility of planktonic cells of *L. monocytogenes* grown in

TSB, concentrations of 50, 100, 200, 300, 400 and 800 parts per million (ppm) were used for E-San®, and concentrations of 70, 200, 500, 800 and 1100 ppm were used for the Perox-E® sanitizer. Working solutions were prepared by diluting the concentrated sanitizers in sterile distilled water and were used within 3 h of preparation. In experiments that tested the susceptibility to sanitizer of *L. monocytogenes* cells grown statically in a biofilm at 35°C for 48 h on the stainless steel coupons, concentrations of 100, 200, 300, 400, 500 and 600 ppm of E-San® were used, and for Perox-E® concentrations of 900, 1100 and 1300 ppm were tested. For experiments that involved biofilms grown on the plastic pegs of a MBEC™ device (MBEC Biofilms Technology Ltd., Calgary, AB, Canada) starting concentrations for E-San® and Perox-E® sanitizers were 10,000 ppm and 38,400 ppm, respectively. These concentrations of sanitizers were added directly to the second column of a 96-well microtitre plate (Nunclon, Delta, Roskilde, Denmark) to be used as a sterility control with no contact with biofilm pegs (i.e. pegs were detached and discarded). Therefore, working solutions of 5,000 ppm E-San® and 19,200 ppm Perox-E were prepared by diluting the stock solutions of sanitizers in TSB. A two-fold dilution series of the working solutions were prepared along the rows of microtitre plates, with the first column containing only TSB as a sterility control without any contact with biofilm pegs and the last column containing TSB in contact with biofilm pegs (positive control) as shown in Figure 3-1.

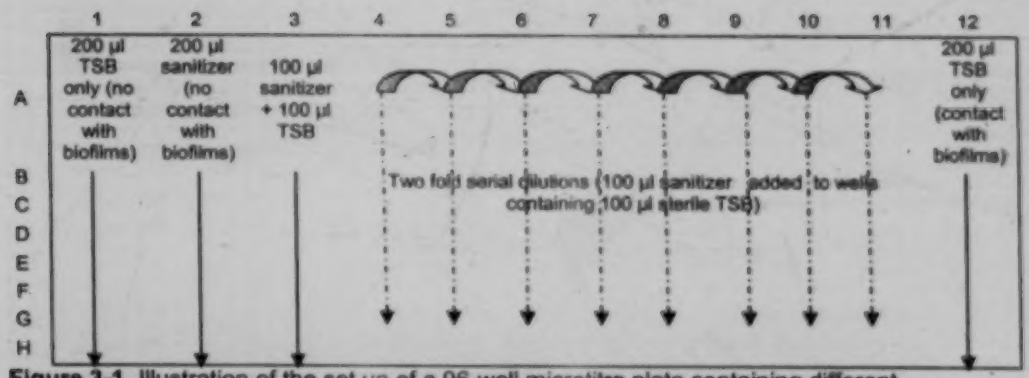

Figure 3-1. Illustration of the set up of a 96-well microtitre plate containing different concentrations of two sanitizers, E-San® (rows A to D) and Perox-E® (rows E to H). One microtitre plate was used for each strain to test the susceptibility to sanitizers of the *L. monocytogenes* grown in the MBEC™ HTP assay.

3.2.4 Microtitre plate assay for biofilm formation on stainless steel coupons

Sterilized coupons were individually placed into the wells of a sterile, polystyrene 24-well microtitre plate (Corning Incorporated, Corning, NY). Biofilms were grown using a new microtitre plate for each strain to minimize the chance of cross-contamination. Once the culture inoculum was standardized to ca. 10^5 CFU/ml, the cultures were vortexed for 15 s and 2 ml of the inoculum was transferred into each well of the microtitre plate that contained a coupon. Plates were covered with a closely fitting lid and incubated statically at 35°C for 48 h. After incubation sterile forceps were used to carefully remove each coupon so as not to disturb the biofilm and the coupon was gently rinsed with sterile distilled water to remove loosely attached cells. The coupons were used immediately in the sanitizer susceptibility assays. For each strain, a negative control (a sterile coupon immersed in 0.85% saline) and a positive control (an inoculated coupon

immersed in 0.85% saline) were run for every experiment. Experiments were replicated at least three times for each strain of *L. monocytogenes*.

3.2.5 Susceptibility of planktonic cells of *L. monocytogenes* to sanitizers

In a sterile 24-well microtitre plate, 1.9 ml of each sanitizer at the appropriate concentrations were added to individual wells, while 0.85% saline was used for the negative and the positive controls, as described previously. Culture inoculum (100 µl) adjusted to ca. 10^7 CFU/ml was added directly to the wells containing appropriate concentrations of sanitizer, gently stirred, and after 30 s 100 µl of the solution was transferred into sterile tubes containing 10 ml of D/E Neutralizing broth (DEB; Difco™). Tubes were incubated at 35°C for 24 and 48 h. Results were recorded as positive when color of the medium turned yellow, and as negative (absence of growth) when color of the medium remained purple. After 48 h, all samples were streaked onto TSA (incubated at 35°C for 24 ± 2 h) to confirm the presence or absence of viable cells. Experiments were replicated at least three times for each strain of *L. monocytogenes*.

3.2.6 Susceptibility to sanitizers of biofilms of *L. monocytogenes* grown on stainless steel coupons

To determine susceptibility of *L. monocytogenes* grown in biofilms on stainless steel coupons, 2 ml of the different concentrations of each sanitizer were added to the wells of a sterile 24-well microtitre plate with 0.85% saline used for the negative and positive controls. Inoculated coupons were lowered into the sanitizer solution with sterile forceps, and allowed contact with sanitizers

for 5 min. Upon removal from the sanitizer solution with sterile forceps, coupons were placed into tubes containing 10 ml of DEB and incubated at 35°C for 24 h and 48 h. Results were recorded as growth (+), absence of growth (-), and unclear (mixed). After 48 h, all samples were streaked onto TSA (incubated at 35°C for 24 ± 2 h) to confirm presence or absence of the viable cells. The assay was replicated at least three times for each strain of *L. monocytogenes*.

3.2.7 Susceptibility to sanitizers of *L. monocytogenes* biofilms grown using the MBEC™ device

Bacterial biofilms of five *L. monocytogenes* strains were grown in the MBEC™ assays following the manufacturer's instructions (Innovotech Incorporated, Edmonton, AB, Canada), and as previously described by Ceri et al. *(5)* with slight modifications. The MBEC™ HTP device used in the experiments consists of a corrugated trough that holds 22 ml of the inoculum and a closely fitting lid with 96 pegs, which allows for growth of one type of biofilm that can be tested using a variety of sanitizers.

The MBEC™ HTP device was positioned on a rocking table (Gel Surfer, Diamed Lab Supplies Inc., Mississauga, ON, Canada) set at 3.5 rpm, at room temperature for 96 h. Following incubation, biofilms formed on the plastic pegs of the MBEC™ device were rinsed with 0.85% saline for 1 min, and then inserted into the microtitre plate containing different concentrations of sanitizers as illustrated in Figure 3-1. The biofilms were exposed to sanitizers for 10 min, under static conditions in a biosafety hood.

Following the exposure to sanitizers, pegs containing the biofilms were rinsed twice in 0.85% saline. After rinsing, biofilms were placed into sterile microtitre plates with each well containing 200 µl of fresh DEB, and disrupted for 15-20 min with a water table sonicator (Bransonic 52, Branson Ultrasonics Corporation, Danbury, CT). Microtitre plates containing DEB and disrupted biofilms were incubated at 35°C for 24 h. Results were recorded based on the color change, as previously described.

Biofilm formation was confirmed prior to exposure to sanitizers by breaking off at least two pegs from the MBEC™ device for each strain and immersing the pegs in 200 µl of 0.85% saline. Biofilms were disrupted by sonication, as described above. Following serial dilution in sterile 0.85% saline, viable counts were determined by spot plating on TSA (incubated at 35°C for 24 h). Viable counts of cells grown for 72 h in TSB (i.e. planktonic cells) in the corrugated trough of the same MBEC™ device used for biofilm growth, were determined by transferring 20 µl of the culture into 180 µl of 0.85% saline, serially diluting and spot plating on TSA (incubated at 35°C for 24 h).

Microtitre plates were used to test the minimal inhibitory concentration (MIC) of each sanitizer against the planktonic cells of *L. monocytogenes*. The MIC was defined as the lowest concentration of the sanitizer in which growth of planktonic bacteria (i.e. loose cells sloughed off from the pegs that remained in the sanitizer solution after 10 min exposure) could not be observed. The MIC values were determined by inspecting wells for turbidity, followed by streaking the

contents of each well onto TSA (35°C for 24 ± 2 h) to confirm presence or absence of microbial growth. The experiments were replicated two times.

3.2.8 Microscopic examination of *L. monocytogenes* biofilms grown on stainless steel coupons

To confirm biofilm formation on the coupons for each of the five strains used in the experiments, following the 48 h growth at 35°C and before any sanitizer treatment coupons were observed using a scanning electron microscope (SEM; Philips/FEI LaB6 Environmental Scanning Electron Microscope). The coupons were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h at room temperature, followed by two rinses with phosphate buffer for 30 min each. The samples were dehydrated in a sequential series of 20, 40, 60, 80% ethanol and two consecutive exposures to 100% ethanol, for 30 min each. The coupons were exposed gradually to hexamethyldisilazane (HMDS) by immersing them for 30 min into each of 3:1, 1:1, 1:3 ratios of ethanol (100%) and HMDS (100%), respectively, followed by two 30 min exposures to 100% HMDS. The coupons were mounted on an aluminum stub and sputter-coated with gold (Hummer Sputter Coater, Anatech, Hayward, CA, U.S.A). The images were captured using the Philips/FEI LaB6 Environmental Scanning Electron microscope operated at 15 kV.

To determine the presence of viable cells after treatment with sanitizers of either 200 or 600 ppm E-San® or 900 ppm Perox-E®, the coupons were stained using LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, Inc., Eugene, OR). The staining solution was prepared by dissolving the contents of

one component A (SYTO 9) dye and one component B (propidium iodide) dye together in 5 ml of sterile distilled water. Final concentrations of the dyes were 6 µM SYTO 9 and 30 µM propidium iodide. The mixed dye (20 µl) was placed on the coupons and left for 15 min in the dark, after which they were viewed using the upright fluorescence microscope (Axio Imager, Carl Zeiss GmbH, Göttingen, Germany) at 100X magnification. A control sample was prepared using a population of 50% live cells of *L. monocytogenes* and 50% dead (exposed to 95°C for 10 min) cells of *L. monocytogenes*.

3.3 Results

3.3.1 Effect of sanitizers on planktonic cells

Among the six concentrations of the E-San® sanitizer that were tested against planktonic cells of different strains of *L. monocytogenes*, the lowest concentration (50 ppm) was effective in completely inactivating cells of all the tested strains after a 30 s exposure time. For Perox-E®, 800 ppm was the minimal concentration required to inactivate the strains of *L. monocytogenes* grown in suspension with a 30 s exposure time.

3.3.2 Effect of sanitizers on biofilms grown on stainless steel coupons

None of the six concentrations of E-San® (100, 200, 300, 400, 500, and 600 ppm) that were used to test the susceptibility of *L. monocytogenes* grown on the stainless steel coupons were effective in complete inactivation of the five strains after 5 min of exposure. However, all three concentrations of Perox-E® (900,

1100, and 1300 ppm) were effective in inactivation of cells grown for 48 h on the stainless steel coupons, after 5 min of contact time. No recovery of injured cells, or microbial growth after incubation of the coupons in neutralizing broth was observed for the lowest concentration of Perox-E® (900 ppm).

Persistent and nonpersistent strains of *L. monocytogenes* did not differ in their susceptibility to different concentrations of the two sanitizers, nor were any differences observed for *L. monocytogenes* ATCC 19115 compared to the persistent and nonpersistent strains of *L. monocytogenes* obtained from the meat processing environment. When grown in TSB *L. monocytogenes* ATCC 19115 required a longer incubation time to reach the same cell density compared to the strains obtained from the environment of meat processing facilities. SEM images revealed lower cell density of cells attached to the coupons after 48 h for the ATCC strain as compared to the two persistent (P1 and P2) and one of the nonpersistent (NP1) strains of *L. monocytogenes* (Figure 3-2).

Following the treatment with sanitizers and 24 h incubation of the DEB, variable results were observed for the *L. monocytogenes* ATCC 19115, where certain localized areas in the DEB were yellow indicating growth and other areas in the broth remained purple. However, after 48 h incubation all samples turned yellow which indicated that the organisms had recovered and were able to grow. In contrast, the bright and distinctive yellow color of DEB was observed for the persistent and nonpersistent strains of *L. monocytogenes* following the 24 h incubation period.

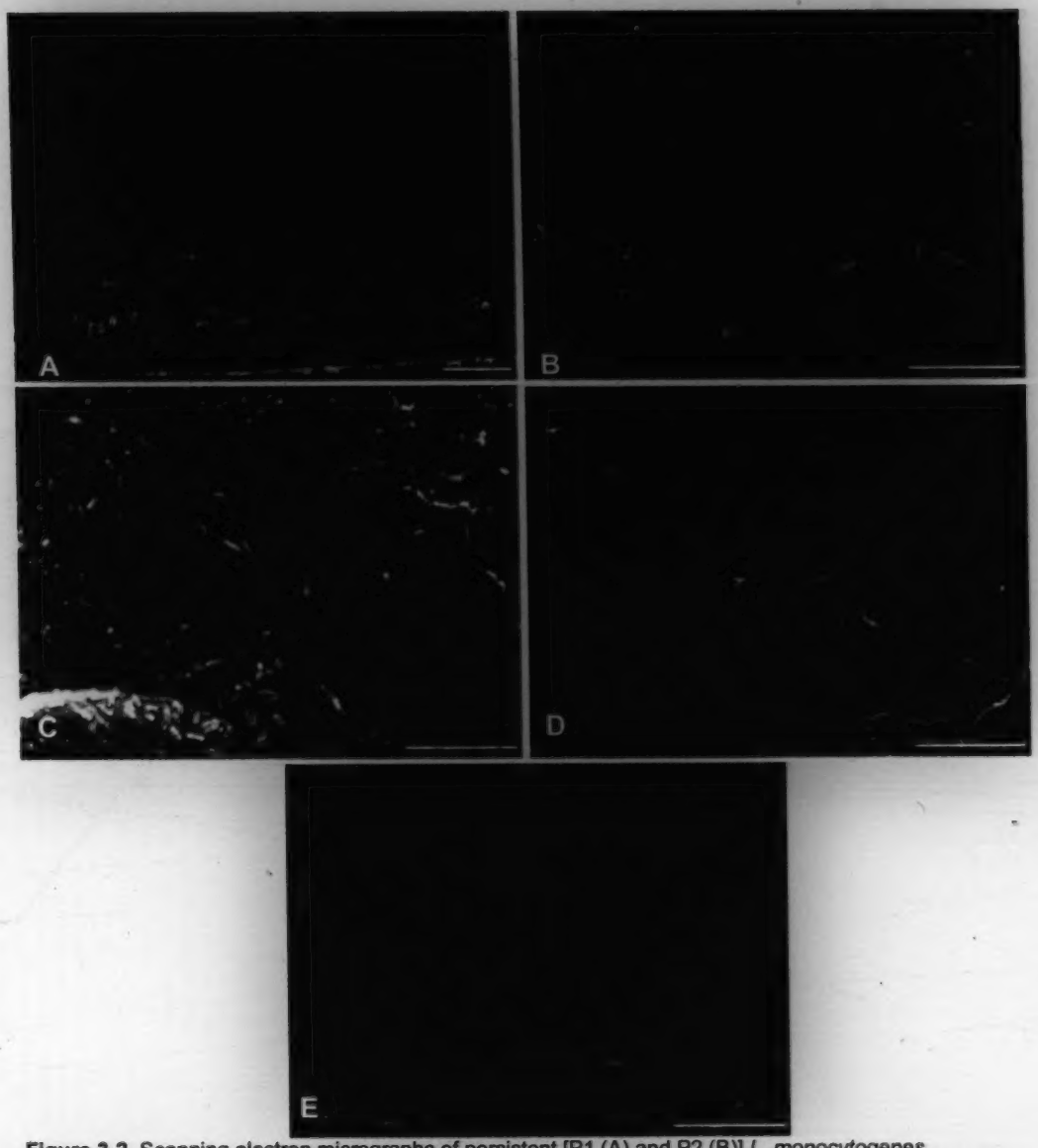

Figure 3-2. Scanning electron micrographs of persistent [P1 (A) and P2 (B)] *L. monocytogenes*, nonpersistent [NP1 (C) and NP2 (D)] *L. monocytogenes* and *L. monocytogenes* ATCC 19115 (E) attached to stainless steel coupons (type 304, No. 4 finish). Persistent and nonpersistent strains were obtained from the environment of a meat processing facility. The bars in the right corner measure 5 µm (A) and 10 µm (B, C, D, E).

Examination of the stainless steel coupons treated with sanitizers and the LIVE/DEAD® *Bac*Light™ probes showed that for all strains not all cells were inactivated after treatment with 600 ppm E-San®, the highest concentration that was tested (Figure 3-3).

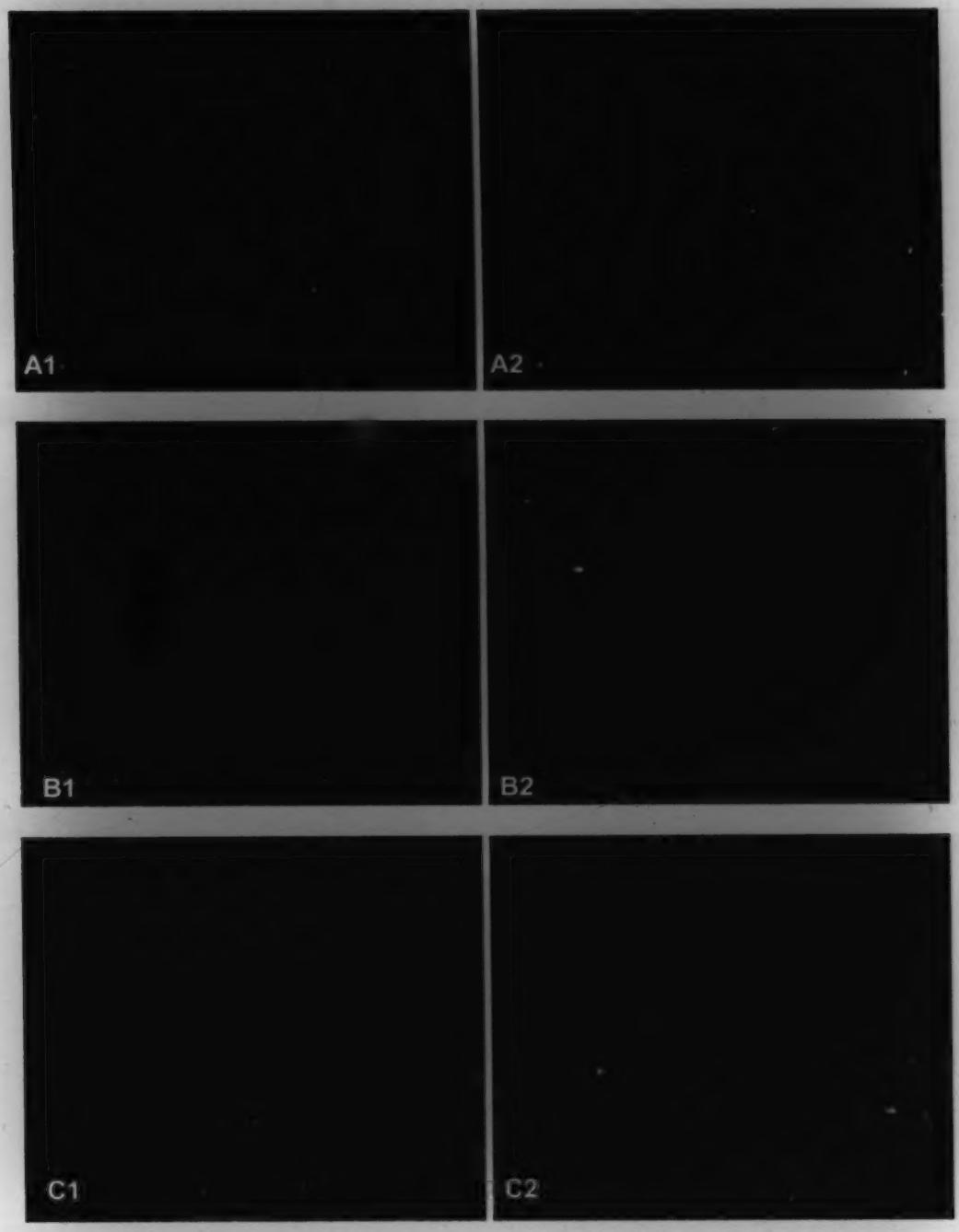

Figure 3-3. Images of persistent P2 (A) and nonpersistent NP2 (B) strains of *L. monocytogenes* obtained from a meat processing facility, and *L. monocytogenes* ATCC 19115 (C) after treatment with 600 ppm quaternary ammonium compound sanitizer (E-San®). Cultures were grown on stainless steel coupons at 35°C for 48 h, treated with sanitizer and stained with LIVE/DEAD® *Bac*Light™ probes. Images A1, B1 and C1 illustrate the population stained with propidium iodide; A2, B2 and C2 show cells stained with SYTO 9.

Results revealed more viable than dead cells when coupons were treated with 200 ppm E-San® (data not shown). When coupons were treated with 900 ppm Perox-E® the cells appeared dead (data not shown).

3.3.3 Effect of sanitizers on biofilm and planktonic cells grown on the MBEC™ devices

The highest concentrations of E-San® and Perox-E® sanitizers tested against biofilms of different strains of *L. monocytogenes* grown for four days at room temperature were 5,000 ppm and 19,200 ppm, respectively. Even at the highest concentration tested, E-San® was not able to inactivate biofilm cells for any of the five strains of *L. monocytogenes* that were tested. The minimal concentration required to inactivate biofilm cells using Perox-E® was 4,800 ppm for all of the five strains of *L. monocytogenes* tested. The Perox-E® sanitizer was more efficient in inactivation of the biofilm cells compared to E-San®; however, both sanitizers were tested at far higher concentrations than those recommended by the manufacturer.

For the planktonic cells of the five strains of *L. monocytogenes* tested the MIC observed for E-San® and Perox-E® were 39 ppm and 150 ppm, respectively. These concentrations are considerably lower than the concentrations recommended by the manufacturer.

3.4 Discussion

Experiments were done to investigate the resistance to two sanitizers of five strains of *L. monocytogenes* grown either as planktonic cells or in biofilms on

stainless steel coupons or plastic surfaces. The minimum inhibitory concentrations of sanitizers for planktonic cells of *L. monocytogenes* were substantially lower than the concentrations recommended for use by the manufacturer. Other researchers *(12)* have also observed that the concentration of sanitizers required to reduce the number of cells grown in liquid medium is much lower than that recommended by the manufacturer. The MICs for the sanitizers used in this study were 5 to 7 times lower than the concentrations recommended by the manufacturers. In the current study 39 ppm QAC (E-San®) or 150 ppm Perox-E® were able to inactivate ca. 10^5 CFU/ml *L. monocytogenes*. Similar results were reported by Earnshaw and Lawrence *(7)* when they exposed 19 different strains of *L. monocytogenes* grown in a suspension to concentrations recommended by the manufacturer for three different sanitizers. The results from the current research are in agreement with the findings of other studies that generally lower concentrations of sanitizers than those recommended by manufacturers are sufficient in inactivation of planktonic cells *(7, 11, 12)*.

Although the concentrations required to reduce populations of *L. monocytogenes* grown as planktonic cells were low, the minimum inhibitory concentrations for the same strains grown on surfaces were far greater than the concentrations recommended by the manufacturer and the concentrations reported in other studies. The investigation of the susceptibility to sanitizers of five strains of *L. monocytogenes* attached to stainless steel and plastic surfaces indicated that resistance of *L. monocytogenes* to QAC is high. When five strains of *L. monocytogenes* were allowed to attach to stainless steel surfaces for 48 h,

the 600 ppm of a QAC sanitizer applied for 5 min was not effective in completely inactivating the population. These results are in accordance with Frank and Koffi *(8)* who reported that microcolonies of *L. monocytogenes* attached to glass surfaces can survive when exposed for 20 min to 800 ppm of a QAC-based and an anionic acid sanitizer. Lee and Frank *(15)* observed 100 times greater resistance to 200 ppm hypochlorite for cells of *L. monocytogenes* allowed to adhere for 8 days to a stainless steel surface when compared to cells grown for 4 h. Other studies have also noted resistance of *L. monocytogenes* cells grown in a biofilm consortium to chlorine-based and iodophor sanitizers *(3, 14, 21)*. However, there are reports that cells of *L. monocytogenes* adhered to surfaces can be effectively inactivated on stainless steel by sodium hypochlorite and QAC-based sanitizers at concentrations recommended by the manufacturers *(22)*. Mustapha and Liewen *(22)* evaluated the ability of two commercially available sanitizers, sodium hypochlorite and a QAC sanitizer, to reduce the population of *L. monocytogenes* ATCC 7644 grown on stainless steel surfaces for 1 h and 24 h. They found that 200 ppm of either sanitizer applied for 2 min could effectively inactivate adherent cells of *L. monocytogenes (22)*. They also reported that 50 ppm QAC applied for 1 min can effectively inactivate cells of *L. monocytogenes* grown on both non-porous and porous stainless steel surfaces *(22)*. These findings are quite contrary to the results reported in the current study. However, some differences in results may be attributed to source variation of the strains of *L. monocytogenes* used in the studies *(25)*, as well as composition of the QAC sanitizer. Mustapha and Liewen *(22)* used *L. monocytogenes* ATCC 7644 while

the strains of *L. monocytogenes* used in the current study were obtained from meat processing environments, where bacteria are known to endure different environmental stressors *(21, 25, 27)*.

The results of the current study indicated that the sanitizer containing hydrogen peroxide and acetic acid was more effective in its ability to inactivate cells grown on stainless steel coupons than the QAC-based sanitizer based sanitizer. In fact, 900 ppm Perox-E® was bactericidal for *L. monocytogenes* biofilms formed on stainless steel. This concentration is less than the concentration recommended by the manufacturer for sanitation of food processing facilities. Other researchers have also reported that acidic QAC, peracetic acid and chlorine dioxide *(14)* can inactivate biofilm cells more efficiently than a neutral QAC *(14)*, chlorine and iodophor *(13)* sanitizer.

Not only differences in sanitizers can impact the inactivation of biofilm cells, but properties of various surfaces have also been demonstrated to play a role in efficacy of sanitizer inactivation of adherent cells *(14, 20, 21, 27)*. In the current study, very high concentrations of the sanitizers were required to inactivate the biofilms of *L. monocytogenes* grown for four days on the plastic pegs of MBEC™ devices. Concentrations of E-San® of 600 ppm and 5,000 ppm were unable to inactivate *L. monocytogenes* biofilms grown for 48 h on the stainless steel coupons and four days on plastic pegs, respectively; whereas 900 and 4,800 ppm Perox-E® effectively inactivated *L. monocytogenes* biofilms grown on stainless steel coupons for 48 hours and plastic pegs for four days, respectively. Mafu et al. *(21)* established that higher concentrations of hypochlorite, iodophors

and a QAC sanitizer are required to inactivate *L. monocytogenes* Scott-A cells attached to polypropylene and rubber surfaces compared to that required to inactivate cells grown on stainless steel and glass surfaces. Polypropylene surfaces are especially difficult to sanitize with concentrations greater than 10,000 ppm hypochlorite and QAC required to inactivate *L. monocytogenes* biofilms *(21)*. Other researchers *(13, 14)* have reported that *L. monocytogenes* grown on plastic or polyester/polyurethane surfaces cannot be easily inactivated. It has been demonstrated that the type of surface can affect the efficacy of a sanitizer and the results from the current study showed that a plastic surface may be more difficult to sanitize than a stainless steel surface. However, differences in assays used to determine sanitizer susceptibility of biofilms grown on stainless steel and plastic surfaces make it difficult to draw clear conclusions that can be applied in the food industry.

Scanning electron micrographs of the different *L. monocytogenes* grown on stainless steel (Figure 3-2) demonstrated the differences in the ability of the different strains to form biofilms. Two persistent strains of *L. monocytogenes* formed biofilms more readily on the stainless steel surface than one of the nonpersistent and the ATCC strain; however one nonpersistent strain was able to form a biofilm that was similar to that of the persistent strains. This is in part in agreement with findings reported by Borucki et al. *(4)*, Norwood and Gilmour *(23)*, and Lundén et al. *(17)* who observed that strains of *L. monocytogenes* that persist in food processing facilities for a long time exhibit higher biofilm formation than the nonpersistent *L. monocytogenes* strains. Even though in the current

study persistent strains of *L. monocytogenes* formed biofilms more readily, there was no difference in the susceptibility of the persistent, nonpersistent and the laboratory control strain of *L. monocytogenes* to sanitizers, which indicated that resistance to sanitizers may not be the reason why some strains persist in food processing environments. Similar conclusions were drawn by Holah et al. *(12)* and Earnshaw and Lawrence *(7)*, who suggested that it is unlikely that persistence of certain strains of *L. monocytogenes* is due to inherent development of sanitizer resistance traits.

In summary, the current research confirmed that planktonic cells of *L. monocytogenes* can be effectively inactivated with concentrations of E-San® and Perox-E® sanitizers lower than those recommended by the manufacturer, regardless of the source of the strain or its persistence in the food processing environment. When strains of *L. monocytogenes* were grown attached to stainless steel and plastic surfaces, higher concentrations of both sanitizers were required for inactivation. The QAC-based sanitizer was not efficient in inactivation of the cells on the stainless steel and the plastic surfaces even with a concentration three and 25 times higher than the manufacturer recommended concentration, respectively. The sanitizer containing peroxide and acetic acid effectively inactivated cells of *L. monocytogenes* grown on stainless steel surfaces. In this case, the concentration of sanitizer required was 1.2 times lower than the concentration recommended by the manufacturer for sanitation of food processing environments. However, to inactivate biofilms grown for four days on plastic surfaces the sanitizer concentration had to be increased to four times

higher than the manufacturer's recommended concentration for application in the food industry. Thus, the choice of a sanitizer can impact the efficacy of bacterial inactivation when microorganisms are part of a biofilm consortium. Even though certain strains of *L. monocytogenes*, in particular those that persist in food processing environments, tend to be better able to form biofilms than those that occur sporadically in the environment, no correlation was observed between strain persistence and resistance to sanitizers when the bacteria were grown in suspension or attached to surfaces. However, it is important to note that only two persistent and two nonpersistent strains of *L. monocytogenes* were used in the current research and that a study with more persistent and nonpersistent *Listeria* spp. may be necessary in order to draw broad conclusions. Simulating the conditions found in a food processing environment is one of the limiting factors in the study of the behavior of biofilm bacteria *(14)*. Therefore, future studies involving multispecies biofilms including a variety of *Listeria* spp. obtained from food processing environments and a variety of biocides used in the food processing sector may give a more complete picture of how bacterial biofilms found in food processing facilities behave when exposed to sanitizers. Additionally, the type of assay used to determine the susceptibility of a population to sanitizers can impact the results, which emphasizes the need for method standardization for assessment of sanitizer susceptibility to allow direct comparison of different studies.

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4. SUMMARY AND CONCLUSIONS

Detection of *Listeria* spp. in food processing environments can be influenced by sampling procedures and detection methods. Findings from the current study demonstrated that the sterile sponge and composite tissue swabbing methods are more efficient in recovery of *Listeria* spp. in samples collected from the food processing areas than the cotton swab sampling method. Similarly, some detection assays may be more efficient in identification of *Listeria* spp. than others. When compared to the conventional culture method, the BAX® method and lateral flow immunoassay were more sensitive and specific than the EL Petrifilm™ detection method for identification of *Listeria* spp. when a large number of samples were collected from meat processing environments. It is believed that *Listeria* spp. are generally present in low numbers in food processing facilities *(2)*. Consequently, if less efficient swabbing and detection protocols are used it is possible that the overall prevalence of *Listeria* spp., and especially *L. monocytogenes (7)*, in food production areas could be underreported.

Investigation of the presence of *Listeria* spp. in two federally inspected meat processing facilities located in Edmonton, AB, Canada, resulted in 0.6% of the samples positive for *Listeria* spp. in one of the facilities and 38% of the samples positive in the other facility. Certain strains of *L. monocytogenes* were repeatedly recovered from the same sampling locations throughout the nineteen-month sampling period, indicating that some strains of *L. monocytogenes*. can adapt to processing conditions and persist in food productions areas for prolonged

periods of time. When two of the persistent strains of *L. monocytogenes* and two strains of *L. monocytogenes* recovered on a sporadic basis from a meat processing establishment were exposed to different concentrations of two sanitizers (E-San® and Perox-E®) no correlation was observed between the susceptibility to sanitizers and strain persistence, regardless of the planktonic or biofilm state. However, the attachment of the cells of *L. monocytogenes* to stainless steel and plastic surfaces impacted sanitizer susceptibility. Higher concentrations of both sanitizers were required to inactivate cells of *L. monocytogenes* grown in biofilms compared to cells of *L. monocytogenes* grown in a liquid suspension. Perox-E® was more efficient in inactivation of biofilms of *L. monocytogenes* on both stainless steel and plastic surfaces compared to E-San®. Based on the results from the current study it can be concluded that the type of sanitizer plays a role in disinfection efficiency when biofilms are present on a surface. Furthermore, the current study demonstrated that concentrations recommended by the manufacturer for a QAC-based sanitizer (E-San®) are inefficient in inactivation of *L. monocytogenes* biofilms grown on stainless steel and plastic surfaces. The sanitizer containing an acidic component and hydrogen peroxide (Perox-E®) at a concentration recommended by the manufacturer had a bactericidal effect on biofilms grown for 48 h on the stainless steel surface. However, higher concentrations of Perox-E® were required for inactivation of *L. monocytogenes* biofilms grown for four days on a plastic surface, which implies that the age of a biofilm and the surface *(3, 4, 6)* type can also affect the sanitizer efficiency. Additional factors such as temperature, static

or dynamic growth conditions, and the history of the bacterial strains may impact biofilm properties and consequently have effect on the susceptibility of biofilm bacteria to sanitizers *(1, 5, 8)*.

In conclusion, complex bacterial communities in the form of biofilms require special attention, especially if these consortia form in food processing facilities and harbor pathogenic microorganisms such as *L. monocytogenes*. The presence of *L. monocytogenes* in food production areas and possible contamination of ready-to-eat foods such as processed meats is of great concern to the food industry, consumers, and the regulatory authorities. In order to achieve successful monitoring and control of pathogenic bacteria such as *L. monocytogenes*, it is important that all steps involved in the testing of food safety are addressed, including sampling methods, detection assays, and validation of cleaning and sanitation protocols.

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